Screening and delineation of molecular mechanisms of action of HbF inducing agents for the treatment of -thalassaemia

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Screening and delineation of molecular mechanisms of action of HbF inducing agents for the treatment of β-thalassaemia

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ABSTRACT

Current agents used for pharmacological reactivation of foetal haemoglobin (HbF) have limited application due to moderate therapeutic properties, variable patient response and potential cytotoxic effects. Therefore, identification of novel HbF inducing agents is still a major research goal to this day.

Identification of new potential HbF inducers has been mainly based on screening of drug libraries. However, this approach has not been very successful in generating new promising agents. In the current project, I employed two approaches for identifying potential HbF inducers: 1) screening of agents that are structurally similar to compounds with known HbF inducing activity; 2) investigating molecular pathways of a known HbF inducer with the aim of identifying suitable targets for therapeutic manipulation and target-based drug design.

The first approach involved screening of eleven xanthines including caffeine and nine hydroxystilbenic derivatives of resveratrol as potential HbF inducers. However, none of the agents had a potent enough HbF inducing activity in order to be considered as promising therapeutic agents. In the second approach, decitabine was chosen based on its high HbF inducing activity and moderate cytotoxicity in K562 cells and primary human erythroid cultures. Chromatin immunoprecipitation was used to characterise epigenetic changes in the β-globin gene locus, and quantitative real-time PCR for investigation of changes in gene expression levels of ten erythroid-related genes, in the presence of the agent. A quantitative iTRAQ proteomic approach coupled with mass spectrometry was used for identification of changes in the proteome of decitabine-treated and un-treated primary human erythroid cultures. The findings suggest that decitabine induces HbF production through activation of signal transduction pathways rather than through hypomethylation of gene promoters. One such possible pathway is the NF-κB pathway. Among the differentially expressed proteins, twenty-seven proteins were associated with the action of decitabine. Two of those proteins, ARHGAP4 and EGLN2, were previously implicated in hydroxyurea-mediated induction of γ-globin gene expression and hypoxia-mediated erythropoiesis, respectively. In addition, the de-ubiquitinating enzyme USP11 was substantially modulated in the presence of decitabine. The exact role of these proteins in γ-globin expression remains to be established.

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1. INTRODUCTION

Red blood cells, the commonest cell type in blood, are responsible for the delivery of oxygen to different tissues around the body. Haemoglobin is the iron-containing protein within the red blood cells that is responsible for the transport and exchange of oxygen. Haemoglobin is a tetrameric protein composed of four polypeptide chains, two α-globin and two β-like globin polypeptide chains encoded by individual specific genes. Mutations in these genes can lead to a quantitative reduction in the respective globin gene or result in the production of haemoglobin variants, referred to as haemoglobinopathies.

1.1. Haematopoiesis and Erythropoiesis

Haematopoiesis refers to the continuous production of cellular blood components from haematopoietic stem cells (HSC). Within the haematopoietic system, HSCs form the top part of the hierarchy and have the ability to give rise to nine different functional cell types through their differentiation properties (Figure 1) (Orkin and Zon, 2008). However, despite the ability of self-renewal, HSCs have low proliferating potential and usually remain in the G_0 phase of the cell cycle (Bradford *et al.*, 1997). The homeostatic control thus relies on the highly proliferating and committed multi-potent (ST-HSC), oligo-potent (CMP, CLP, GMP, MEP) and lineage-restricted progenitor cells (Pro-T, Pro-NK, Pro-B, Pro-DC, erythroblasts, megakaryocytes) within the haematopoietic hierarchy (Bryder *et al.*, 2006).

In mammals, the location of haematopoiesis changes during development, starting in the yolk sac followed by sequential migration to the intraembryonic aorta-gonad-mesonephros (AGM) region, foetal liver, thymus, spleen and finally bone marrow. The initial production of HSCs in yolk sac is known as "primitive erythropoiesis", but it is only transient and is rapidly replaced by adult-type haematopoiesis known as "definitive haematopoiesis". The AGM region produces the first adult type HSCs at around E11 in embryonic mice (Muller *et al.*, 1994). Definitive haematopoiesis then continues with colonisation of HSCs in the foetal liver which serves as a temporary haematopoietic territory during the foetal stages of development until birth when the haematopoietic cells again migrate and colonise the bone marrow. It has been suggested that none of these sites can generate HSCs *de novo*, but rather expand populations of HSCs that have migrated to these sites (Houssaint, 1981). Moreover, HSCs have also been located in the

placenta around the same time as HSCs appear in AGM (Gekas *et al.*, 2005). However, their exact role in haematopoiesis is still unknown.

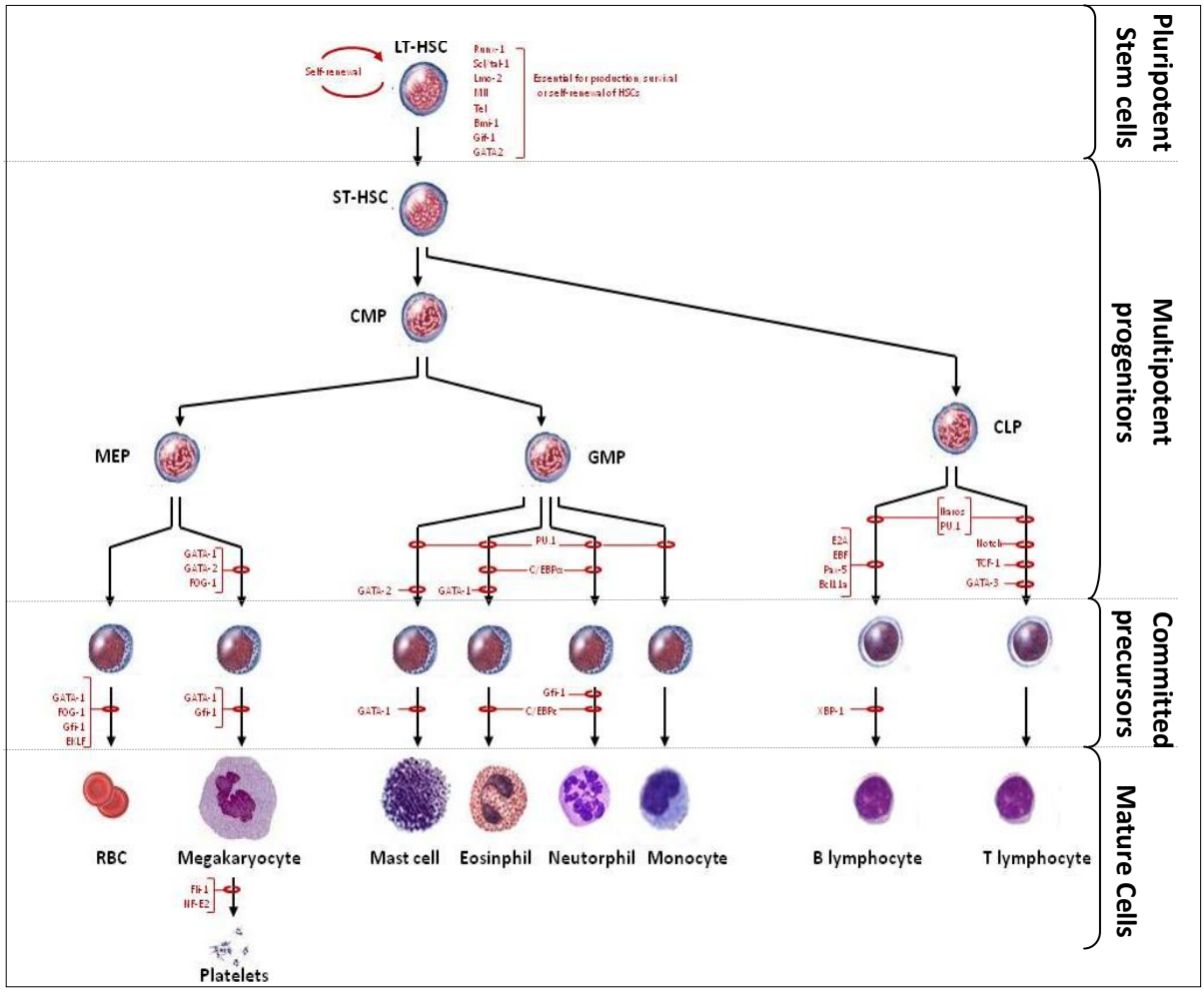


Figure 1, Haematopoiesis. All progenitors originate from pluripotent stem cells that have the ability for self renewal. Except for lymphocytes, all white and red blood cells originate from a single progenitor from the myeloid lineage. Abbreviations: LT-HSC, long-term haematopoietic stem cell; ST-HSC, short-term haematopoietic stem cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythroid progenitor; GMP, granulocyte/macrophage progenitor; RBCs, red blood cells. Adapted from *Orkin and Zon (2008) Cell 132(4):631-644* and *http://stemcells.nih.gov/info/basics/pages/basics4.aspx*

Adult haematopoiesis begins with the HSC and proceeds unidirectionally in a primarily irreversible process under normal physiological steady-state conditions. HSCs form multipotent progenitor cells that retain full lineage potential while having limited

capacity for self-renewal. Multipotent progenitors then give rise to the oligopotent common lymphoid progenitor and common myeloid progenitor which have a more restricted developmental potential. Such oligopotent progenitors in turn give rise to more lineage-restricted progenitors from which all the mature blood cells arise (Bryder *et al.*, 2006) (Figure 1).

Mature enucleated erythrocytes are produced from such lineage-restricted erythroid progenitors in a process known as erythropoiesis. Primitive erythrocytes, originating in the blood island of the yolk sac, are relatively large cells characterised by the expression of embryonic globins and are the only erythrocytes that retain their nucleus when entering the circulation. During definitive erythropoiesis, enucleated erythropoietic cells are produced initially in foetal liver and later in blood, replacing the embryonic red blood cells. Near the time of birth, the site of erythropoiesis switches to the bone marrow and spleen, with the bone marrow being responsible for the steady-state regulation of adult erythropoiesis (Figure 2).

The earliest recognizable erythroid committed progenitors are the proerythroblasts which are identified in the bone marrow. When stained with Giemsa or Wright stain, proerythroblasts appear as large cells with dark-blue cytoplasm and a central nucleus with slightly clumped chromatin. By a series of progressive cell divisions, proerythroblasts give rise to basophilic, polychromatic and orthochromatic erythroblasts which accumulate progressively more haemoglobin and decrease in size while the nuclear chromatin becomes more condensed and the RNA and protein producing apparatus are removed from the cytoplasm (Figure 3). Although protein synthesis is active throughout the erythroid maturation, haemoglobin production is activated at the polychromatic normoblast stage and continues to the reticulocyte stage of development after which no further protein synthesis occurs in red blood cells. RNA synthesis continues up to the orthochromatic stage after which nucleotide synthesis ceases. Similarly DNA synthesis is active in early stages and but not in the late polychromatic and orthochromatic erythroblasts (Borsook, 1964, Weatherall, 1974). When the nucleus is finally extruded from the erythroblasts, reticulocytes are formed within the bone marrow and are still able to synthesise haemoglobin and still contain some ribosomal RNA. Reticulocytes are slightly larger than mature red cells and they spend 1-2 days in the marrow.

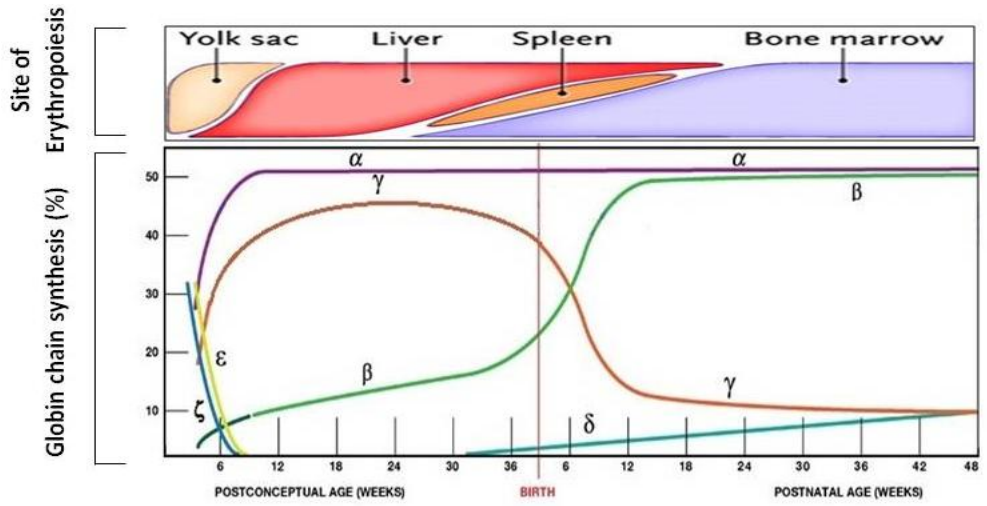


Figure 2, Developmental regulation of globin gene expression during ontogeny. The site of erythropoiesis changes during development (top panel) while the levels of expression of embryonic (ζ & ε), foetal (α & γ) and adult (α & β) globin chains vary at different gestational ages. Adapted from the *www.medicalbiochemistrypage.org*

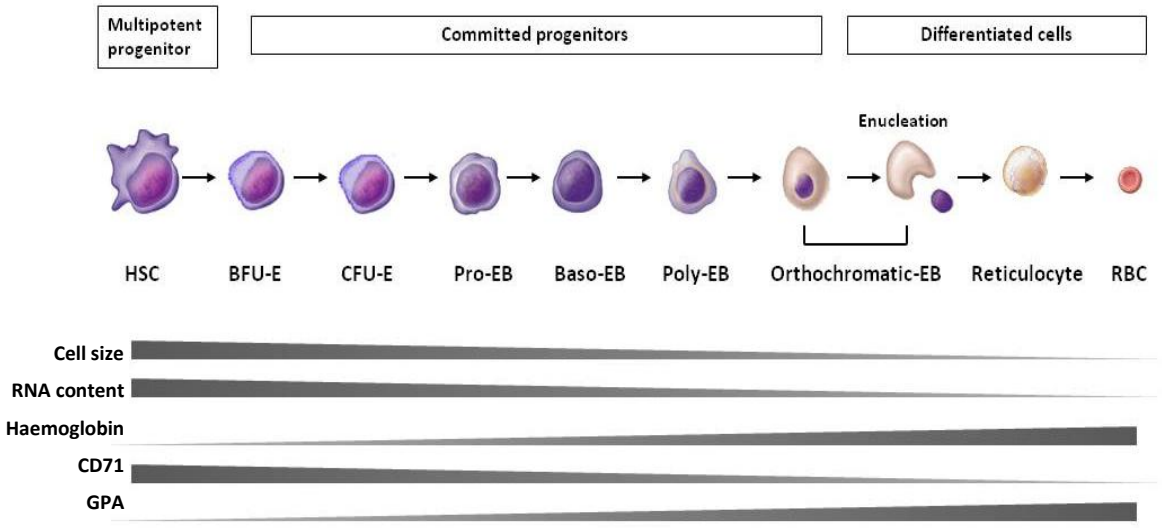


Figure 3, Erythropoiesis. During foetal development, HSCs migrate to the bone marrow that constitute the main site of erythropoiesis for the rest of the adult life. The earliest recognisable erythroid progenitors are the proerythrolasts which are identified in the bone marrow. By a series of progressive cell divisions, proerythroblasts (Pro-EB) give rise to basophilic (Baso-EB), polychromatic (Poly-EB) and orthochromatic erythroblasts which accumulate progressively more haemoglobin and reduce in size while the nuclear chromatin becomes more condensed and the RNA and protein producing apparatus are removed from the cytoplasm. Early progenitors express high levels of proliferation markers (CD71) and minimal levels of differentiation markers (GPA), whereas the differentiated cells have low proliferation and high differentiation markers. Adapted from *http://mahmrabeh.weebly.com/haematology---605-zoo.html.*

Once they are released into the circulation, they circulate in the peripheral blood for 1-2 days before they mature, mainly in the spleen, with all the RNA being lost resulting in the formation of a non-nucleated biconcave disc known as the mature red blood cell (Figure 3).

Schofield (1978) was the first to introduce the importance of stem cell microenvironment, the niche that can extrinsically influence stem cell behaviour. Subsequent studies have confirmed the importance of localised extracellular signals in regulating stem cell self-renewal and differentiation. Experiments in genetically modified mice have shown that HSCs reside adjacent to osteoblasts in the periosteal region of the bone marrow suggesting an important role of osteoblasts in the HSCs niche (Nilsson *et al.*, 1997).

Other studies have demonstrated that the HSCs reside next to vascular endothelium (Kim *et al.*, 2005) suggesting that different anatomical locations represent different niches for HSCs, that can have a different impact on regulation of self-renewal or lineage outcome. For example, HSCs in foetal liver are proliferating while the HSCs in bone marrow are mostly quiescent (Orkin and Zon, 2008).

In addition, the microenvironment contains factors that trigger developmental signalling pathways and activate genetic programs to direct haematopoietic development and lineage-restricted differentiation. Induction events are orchestrated by signalling pathways that turn on or off transcription factors that regulate the expression of specific panels of genes associated with haematopoietic fate and function (Figure 1). Remarkably, the majority of transcription factors in the haematopoietic system are involved in chromosomal translocation and somatic mutations in human haematopoietic malignancies. Stem cell leukemia (SCL)/ T-cell Acute lymphocytic leukemia 1(TAL-1) and LIM domain only 2 (LMO2) are among the transcription factors that are essential for development of both the primitive and definitive haematopoietic systems (Porcher *et al.*, 1996, Gering *et al.*, 1998) while Runx1 is important for developmental specification of HSCs' fate (Burns *et al.*, 2005). Therefore, HSCs can be described as groups of cells with varying developmental potential based on intrinsic networks driven by transcription factors and inputs from the cellular niches in which they reside (Orkin and Zon, 2008).

1.2. Regulation of globin gene expression

In humans, globin gene expression is characterised by two different developmental switches, the embryonic switch and the foetal switch (Thein, 2004). These switches coincide with changes in the morphology of the erythroid cells, the site of erythropoiesis and haemoglobin composition (Figure 2). At approximately 6 to 8 weeks of gestation, the expression of the embryonic ε-globin gene switches to that of the two γ-globin ($^{A}\gamma$ and $^{G}\gamma$) genes in the foetal liver (embryonic switch). This involves the silencing of the ε-globin gene and up-regulation of γ-globin expression (85-98%) which is maintained throughout foetal life (Thein, 2004, Stamatoyannopoulos, 2005, Testa, 2009). Foetal haemoglobin (HbF, $\alpha_2\gamma_2$) has a relatively high affinity for oxygen compared to adult haemoglobin, a property that favours the exchange of oxygen between adult haemoglobin in the maternal red blood cells and foetal haemoglobin in foetal blood through the placenta (Gambari and Fibach, 2007). Shortly after birth, γ-globin expression is almost completely silenced (<5%) while the expression of β- and δ-globin genes is up-regulated in a process known as foetal switching (Testa, 2009) (Figure 2). Expression of the γ-globin gene continues to decrease but is not silenced, with foetal haemoglobin reaching less than 1% of the total haemoglobin at around 2 years of age (Rutland *et al.*, 1983). In adult blood, the dominant haemoglobin is adult haemoglobin (HbA, $\alpha_2\beta_2$) that comprises about 95% of the total haemoglobin. Each globin chain binds to a haem molecule via a histidine side chain which consists of a charged iron atom in the centre of a large heterocyclic organic ring known as porphyrin (Perutz, 1976). In addition to the residual levels of HbF which reside in a subset of erythrocytes known as F cells (Boyer *et al.*, 1975, Thein and Menzel, 2009), normal adult blood contains 2.5-3.5% HbA_2 ($\alpha_2\delta_2$) (Kunkel *et al.*, 1957).

The genes responsible for the synthesis of globin chains are clustered in two regions (Figure 4). The α-like globin gene locus on the tip of chromosome 16, consists of three genes; α1, α2 and ζ genes (Deisseroth *et al.*, 1977, Lauer *et al.*, 1980). The β-like globin genes are found in another locus, a 100kb region on chromosome 11 that consists of five functional genes, ε, $^{G}\gamma$, $^{A}\gamma$, δ and β (Fritsch *et al.*, 1980). The globin genes are arranged in the order in which they are expressed during development. The two γ-globin genes are the result of a 5-kb tandem duplication, and their coding sequences differ only by a single nucleotide within codon 136 in exon 3 (Schroeder *et al.*, 1968), giving rise to $^{G}\gamma$ and $^{A}\gamma$ respectively. In adults, HbF is composed of 40% $^{G}\gamma$ and 60% $^{A}\gamma$ whereas in

the newborn HbF is composed of 70% $^{G}\gamma$ and 30% $^{A}\gamma$. At the molecular level, all the globin genes consist of three exons separated by two intervening regions or introns (Testa, 2009, Thein, 2004).

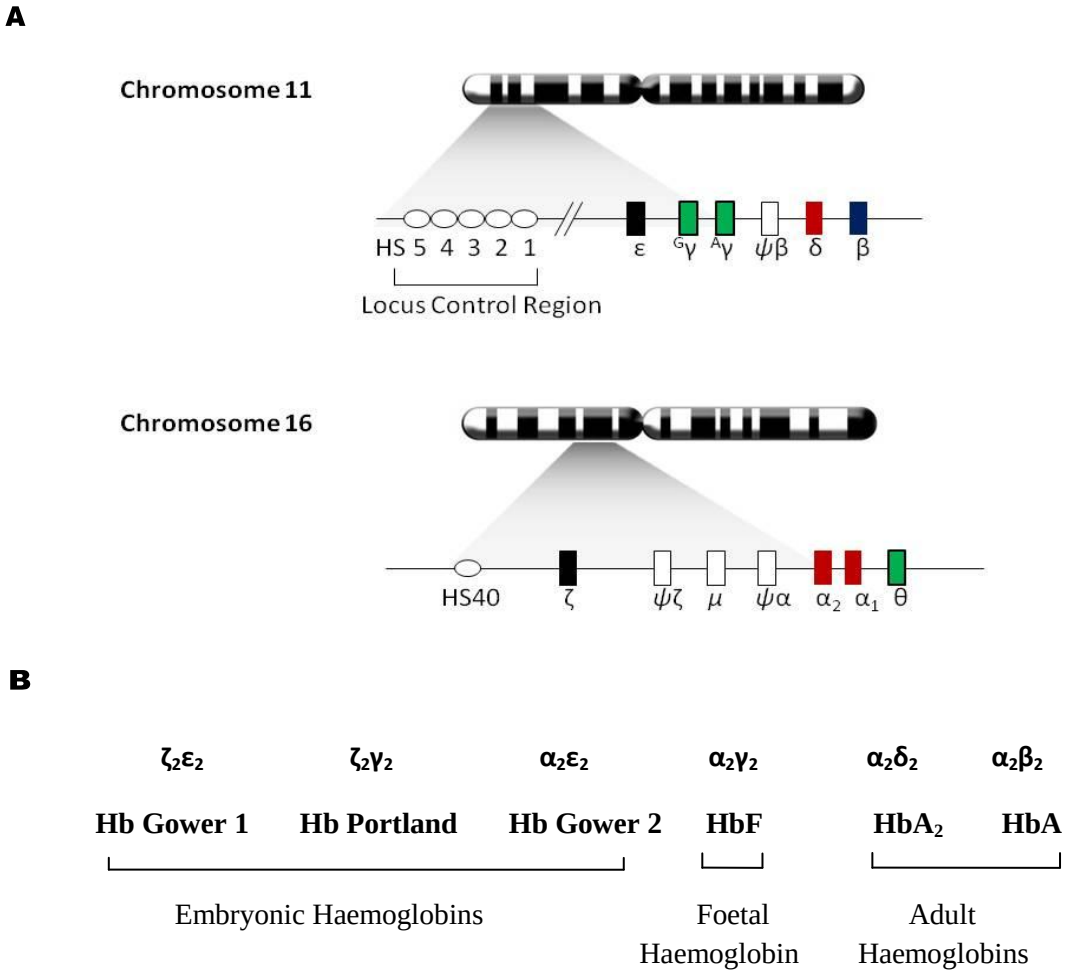


Figure 4, Haemoglobin expression. (A) Genes responsible for the expression of globin chains are clustered into two regions, the α-like globin gene locus on chromosome 16 and β-like globin gene locus on chromosome 11. The genes are arranged in the order they are expressed, leading to the formation of the different types of haemoglobins at different stages of development (B). Adapted from *http://itg.content-e.eu/Generated/pubx/173/hematology/sickle_cell_anaemia.htm*

Evidence suggests that switching between the expression of different globins relies on two mechanisms, gene silencing and gene competition. Expression of individual genes relies on direct physical interaction between the globin gene promoters and *cis*-acting elements such as the Locus Control Region (LCR), mediated by transcription factors

and chromatin remodelling activities. While the ε- and γ- globin genes are autonomously silenced at the appropriate developmental stage, expression of the adult β-globin gene (*HBB*) depends on the lack of competition between the γ-gene for the LCR sequence and direct interaction of *HBB* itself to the LCR. In addition, the restricted expression of the ε-globin gene in yolk sac when placed close to the LCR suggests that ε-globin gene expression is depended on the LCR (Raich *et al.*, 1990). Mutations of GATA1, YY1 or Sp1 binding sites within the ε-promoter abolished ε-globin gene silencing suggesting that silencing of the embryonic gene might be combinatorial, requiring multiple transcription factors for the formation of the silencing complex (Raich *et al.*, 1995). It is currently assumed that the silencing complex turns off ε-globin gene expression in the definitive erythropoiesis by inhibiting the interaction between the ε-globin gene and the LCR (Stamatoyannopoulos, 2005).

Silencing of the γ-globin gene appears to involve both autonomous silencing (Dillon and Grosveld, 1991) and gene competition (Enver *et al.*, 1990). The presence of developmental expression of γ-globin gene in transgenic mice carrying βYAC construct with a deleted β-globin gene suggests that the γ-globin gene is mainly regulated by autonomous silencing (Peterson *et al.*, 1995). In addition, deletion of the -378 to -730 region of the γ-promoter leads to continuous expression of the γ-globin gene suggesting the presence of a silencer within this deleted stretch of DNA upstream of the γ-globin gene (Stamatoyannopoulos *et al.*, 1993). Although autonomous silencing appears to be the principal mechanism that switches off the expression of the foetal globin gene, increased γ-globin gene expression in patients with β-thalassaemia due to β-promoter deletions suggests that competition with the β-globin gene promoter might also contribute to γ-globin gene silencing (Stamatoyannopoulos, 2005). Gene competition between the β- and γ-globin gene has also been suggested after observations that developmental regulation is lost in transgenic mice carrying either the γ- or β-globin gene close to the LCR (Enver *et al.*, 1990, Behringer *et al.*, 1990). *In situ* hybridization methods have shown that the LCR interacts with only one promoter in the globin locus at a given time (Wijgerde *et al.*, 1995), while more advanced techniques such as 3C and RNA trap assay, have shown that transcribed globin genes are in close proximity to the LCR, confirming the "looping model" of globin gene expression (Tolhuis *et al.*, 2002) (Figure 5). Loop formation requires the presence of *trans*-activating erythroid specific transcription factors such as EKLF (also known as KLF1) (Drissen *et al.*, 2004),

GATA1 and FOG-1, where binding within *cis* elements presides loop formation (Vakoc *et al.*, 2005).

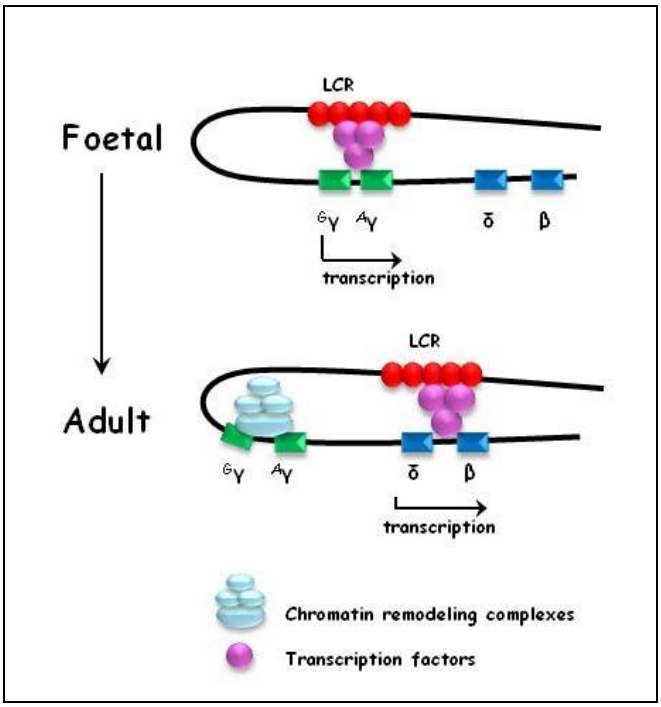


Figure 5, The 'looping model' for globin gene expression. The model aims to explain haemoglobin switching and is based on the direct interaction of the LCR with the individual globin gene promoters. In the presence of *trans*-activating transcription factors, the LCR comes in close proximity to the promoters of γ-globin genes at the prenatal age, leading to the expression of foetal haemoglobin. Shortly after birth, the presence of chromatin remodelling complexes and *trans*-activating transcription factors shifts the interaction of the LCR with the β-globin promoter leading to the expression of adult haemoglobin.

1.2.1. *Cis*-regulation

At least three different *cis*-acting DNA elements have been shown to have a role in the transcription of eukaryotic genes including core promoters, proximal regulatory elements, and distal regulatory elements. These *cis*-acting transcriptional regulatory elements contain recognition sites for *trans*-acting DNA–binding transcription factors that either enhance or repress transcription (Maston *et al.*, 2006).

There are four motifs commonly associated with core gene promoters, the TATA box along with the TFIIB-recognition element (BRE), the Initiator element (Inr) and Downstream Promoter Element (DPE) motifs. In contrast to most gene promoters which contain CpG islands with no obvious TATA box, globin gene promoters contain well-defined TATA boxes without any CpG islands nearby. Mutations in all the positions of the TATA box have been associated with β-thalassaemias while mutations within the DPE and Inr motifs have also been associated at least once with β-thalassaemia. The upstream regulatory region adjacent to the basal promoters contains a CCAAT box

(Dierks *et al.*, 1983), a conserved motif that allows binding of NF-Y and CP1 proteins (deBoer *et al.*, 1988). In addition, two upstream regulatory elements were identified in the globin promoters and include a CCAAT box which acts as a binding site for transcription factors of the Krüppel-like zinc finger class (KLF), and GATA1 binding sites which are implicated in the positive regulation of GATA1 gene and related proteins. Phylogenetic footprinting led to the discovery of the stage selector element (SSE) in the human γ-globin gene promoter, a binding site for a factor implicated in the differential expression of γ-and β-globin genes (Jane *et al.*, 1992). In contrast to the β-like globin genes, α-globin gene promoters are composed of CpG islands and do not contain binding sites for GATA1. The regulated expression of α-globin in the presence of CpG islands suggested that CpG might be a key component of the *cis*-regulatory elements for the α-globin gene in humans (Shewchuk and Hardison, 1997).

Earlier experiments demonstrated the presence of two proximal enhancers around the β-globin gene, one within the gene and another downstream of the gene (Behringer *et al.*, 1987, Trudel and Costantini, 1987), and one at around 1kb downstream of the polyadenylation signal of the ^{A}γ-globin gene (Bodine and Ley, 1987). Both enhancers of the β-globin gene are implicated in the expression of the gene in primates and contain binding sites for GATA1 (Wall *et al.*, 1988). The γ-globin gene enhancer was mapped to a tissue-specific DNase I hypersensitive site and was the only DNA segment surrounding the γ-globin genes that could enhance reporter gene expression when driven by the γ-globin gene promoter in transfected erythroid cells (Bodine and Ley, 1987).

One important aspect that is commonly found in the α-like and β-like globin gene clusters is the presence of strong distal enhancers. Tuan *et al.* (1985) identified five DNAse I hypersensitive sites (HS) located 6 to 20kb upstream (5') of the β-globin which were shown to be involved in embryonic, foetal and adult globin gene expression, now referred to as the locus control region (LCR) (Forrester *et al.*, 1987). Each HS comprises of approximately a 250bp long sequence devoid of nucleosomes which is composed of arrays of multiple ubiquitous and lineage-specific transcription factor-binding sites (Li *et al.*, 2002, Stamatoyannopoulos, 2005). HS1-4 are erythroid specific and act as enhancers of the β-globin LCR while HS5 is found in cells of multiple lineages and acts as an insulator (Li and Stamatoyannopoulos, 1994). HS2 is the most prominent enhancer element in the β-globin LCR as shown by close proximity

of HS2 to the actively transcribed β-globin gene (Carter *et al.*, 2002). Moreover, experiments in transgenic mice (Roberts *et al.*, 1997) and human erythroid cells (Sargent *et al.*, 1999) showed a switch between the contacts of HS2 and HS3 with embryonic and foetal globin genes in yolk sac to contacts with adult globin genes in adult erythroid cells, confirming that interactions between the LCR and the various genes of the β-globin locus are developmentally controlled (Fraser *et al.*, 1993). Almost all HS contain three transcription factor–binding motifs; Maf-response elements (MARE) to which transcription activator proteins of the basic leucine zipper class can bind, GATA motifs and CACC motif to which a family of zinc-finger proteins including KLF1 can bind (Ney *et al.*, 1990, Philipsen *et al.*, 1990, Talbot *et al.*, 1990, Strauss and Orkin, 1992, Andrews *et al.*, 1993). HS2 of the β-globin LCR also contains three E-boxes which act as binding sites for TAL-1 and its heretodimeric partners involved in haematopoiesis (Elnitski *et al.*, 1997).

For the α-globin cluster, there are four highly conserved, noncoding sequences called multispecies conserved sequences (MCS) R1-R4 which act as regulatory elements. The MCS R1, R2, R3 and R4 sequences were previously identified as DNase I hypersensitive sites and referred to as HS-48, HS-40, HS-33 and HS-10, respectively, corresponding to their position relative to the ζ-globin mRNA cap site. However, only HS-40 (MCS R2) seems to be essential for α-globin expression (Higgs and Wood, 2008).

1.2.2. *Trans*-regulation

Trans-regulation is mediated by nuclear DNA-binding proteins that recognise *cis*-acting regulatory elements. These transcription factors can enhance or inhibit gene expression by modifying chromatin structure either by direct binding on proximal or distal regulatory elements or by assembling multiprotein complexes that establish and maintain active or repressive chromatin states. This is facilitated by the formation of long-range chromatin loops which bring critical regulatory elements into physical proximity to the gene promoters.

1.2.2.1. Erythroid-specific transcription factors

GATA1 is the founding member of the GATA family of proteins that comprise of zinc finger transcription factors. It was originally identified as a protein with binding specificity for the β-globin 3' enhancer (Wall *et al.*, 1988). Whole genome occupancy maps developed by multiple groups showed that only a minority of the GATA1 binding sites are located at proximal promoter regions close to the transcription initiation site while the majority occur at distant regulatory elements with equal distribution between intra- and intergenic regions (Fujiwara *et al.*, 2009). GATA1 is essential for erythroid cell maturation, erythroid cells from *gata1*-deficient mice fail to mature beyond the proerythroblast stage (Pevny *et al.*, 1995), while *gata1*-null mouse embryos die from severe anaemia between E10.5 and E11.5 (Fujiwara *et al.*, 1996). The arrest in primitive and definitive erythropoiesis of embryonic stem cells lacking *gata1* beyond the proerythroblast stage can be attributed to the apoptosis of the precursors (Weiss and Orkin, 1995). GATA1 is directly involved in cell survival due to its ability to activate erythropoietin (EPO) receptors (Zon *et al.*, 1991), an important signalling pathway for survival of erythroid progenitors, as well as its ability to alter the expression of Bcl-X_L (Gregory *et al.*, 1999), a gene encoding an anti-apoptotic protein. A direct role of GATA1 in the regulation of foetal to adult switch in humans has been proposed by the identification of patients with congenital erythropoietic porphyria and elevated HbF who had a GATA1 zinc-finger mutation (Phillips *et al.*, 2007). GATA1 appears to facilitate chromatin loop formation at both the c-kit and β-globin loci (Jing *et al.*, 2008) through interactions with erythroid-specific and ubiquitous transcription factors. GATA1 interacts with the Friend of GATA1 (FOG-1) through its N-terminal finger, an interaction that is important for erythroid and megakaryocytic development (Tsang *et al.*, 1997). In fact, GATA1 was shown to bind to a region upstream of both γ-globin gene promoters in a FOG-1 dependent manner which leads to recruitment of the repressive Nucleosome Remodelling Deacetylase (NuRD) complex (Harju-Baker *et al.*, 2008), suggesting a role in foetal to adult globin switching. Functional assays have shown GATA1 to physically interact with KLF1, Sp1 (Gregory *et al.*, 1999) as well as with components of the basal transcription factor complex including p300 and CREB-binding protein (CBP). The interaction leads to acetylation of GATA1 itself (Kasper *et al.*, 2002) and recruitment of CBP to activated genes and erythroid specific transcription factors. More recently, miR-144/451 locus was shown to be a direct target of GATA1

and mice lacking miR-144/451 or miR-451 alone show impaired erythropoiesis, particularly under stress conditions (Dore *et al.*, 2008).

In erythroid cells, SCL/TAL1 transcription factor forms a complex with the basic helix-loop-helix protein E2A and with the LIM domain (domains originally identified in Lin11, Isl-1 and Mec-3 proteins)-containing cofactors LIM domain only 2 (LMO2) and LIM domain-binding protein 1 (LDB1), which in turn interact with GATA1 to form a pentameric complex that binds to a short consensus DNA motif known as the E-box (Wadman *et al.*, 1997). LDB1 directly binds to DNase-hypersensitive sites HS2, HS3 and HS4 of the β-globin LCR and forms long-range interactions with the β-globin promoter, despite the absence of a functional LDB1 binding site at the β-globin promoter (Soler *et al.*, 2010). Expression of SCL/TAL1 coincides with GATA1 expression in erythroid cells and is highly expressed in erythroid precursors and erythroid cell lines (Green *et al.*, 1991, Visvader *et al.*, 1991). Forced expression of the SCL in human $CD34^+$ cells stimulates formation of erythroid and megakaryocyte progenitors and an increase in the size of erythroid colonies (Elwood *et al.*, 1998). Disruption of the SCL/TAL1 gene in adult haematopoietic stem cells causes erythroid and megakaryocytic defects suggesting a role of the SCL factor in the onset of haematopoiesis (Mikkola *et al.*, 2003). Moreover, SCL/TAL1 can activate and repress transcription through recruitment of coactivators such as p300/CBP (Huang *et al.*, 1999) or corepressors such as Sin3A or Eto-2 (Schuh *et al.*, 2005).

Nuclear factor-erythroid 2 (NF-E2) was first described as an activating protein-1 (AP-1) –like DNA-binding factor required for proper function of the erythroid-expressed promoter of the porphobilinogen deaminase gene (Mignotte *et al.*, 1989). NF-E2 binds on the AP-1-like motifs (MAREs), important *cis*-elements within the HS2 of the β-globin LCR (Ney *et al.*, 1990), that lead to formation of DNA hypersensitive sites (Stamatoyannopoulos, 2005). NF-E2 binding sites were also found within the α-globin LCR (Jarman *et al.*, 1991). NF-E2 is a heterodimeric protein composed of two basic-leucine zipper DNA-binding polypeptides, the haematopoietic-restricted p45 subunit (Ney *et al.*, 1993) and the non-haematopoietic specific subunit, p18 (Andrews *et al.*, 1993). p45 was suggested to have a critical regulatory role in globin gene expression due to its ability to restore α- and β-globin gene expression in p45 transduced murine erythroleukaemia cells that lacked both alleles of p45 (Lu *et al.*, 1994). However, targeted inactivation of p45 in mice did not disrupt erythropoiesis and globin gene

expression, but rather severely affected megakaryocyte maturation (Shivdasani and Orkin, 1995). Similarly, p18 deficient mice developed normally and displayed no obvious defects in erythropoiesis, globin gene expression and platelet maturation (Kotkow and Orkin, 1996). However, the N-terminal domain of p45, which was found to be crucial for the NF-E2 activity, was shown to interact with other proteins including CBP/p300 (Cheng *et al.*, 1997), ubiquitin ligase (Gavva *et al.*, 1997) and the TBP-associated factor TAFII130 (Amrolia *et al.*, 1997), suggesting a chromatin modifying role for NF-E2 through interaction with the basal transcription machinery and recruitment of CBP/p300 associated histone acetyltransferases to the LCR.

Kruppel-like factor 1 (KLF1), previously known as erythroid Kruppel-like factor, (EKLF) is a zinc finger transcription factor with three highly similar C-terminal C2H2-type zinc fingers that acts as an activator of the adult β-globin gene through a highly conserved CACCC motif (Miller and Bieker, 1993). KLF1 is restricted to erythroid cells leading to embryonic lethality in *klf1*-null mice (Perkins *et al.*, 1995). Although, it was initially thought that KLF1 was an adult-stage specific factor that facilitates foetal to adult haemoglobin switching, other studies have shown that an LCR-β-globin transgene normally expressed in primitive erythroid cells is not expressed in the absence of KLF1 (Tewari *et al.*, 1998), suggesting a role of KLF1 in regulating both definitive and primitive erythropoiesis. Borg *et al.* (2010) identified a nonsense mutation in KLF1 in a Maltese family with hereditary persistence of foetal haemoglobin (HPFH) which suggested that KLF1 is an activator of BCL11A. Independently, Zhou *et al.* (2010) demonstrated a role of KLF1 as a repressor of foetal globin gene and activator of BCL11A. KLF1 was shown to interact with HS1-3 of the human β-globin LCR (Im *et al.*, 2005) and was able to physically and functionally interact with CBP/p300 (Zhang *et al.*, 2001) and BRG1 (Armstrong *et al.*, 1998). Studies has shown that KLF1 promotes the formation of a high-order chromatin loop at the β-globin locus (Drissen *et al.*, 2004) and functions as a transcriptional repressor by recruiting Sin3A and histone deacetylase 1 (HDAC1) (Chen and Bieker, 2001). Within the KLF family, KLF3 is a repressor of embryonic globin genes as shown by the derepression of the embryonic globin genes in *klf3*-deficient mice (Funnell *et al.*, 2013).

1.2.2.2. Ubiquitous factors involved in globin gene switching

Genome wide association studies showed that B-cell lymphoma/leukemia 11A (*BCL11A*) gene on chromosome 2 is associated with HbF levels in non-anaemic individuals (Menzel *et al.*, 2007). The zinc finger transcription factor, which was initially identified as myeloid or B cell proto-oncogene in humans, exhibited stage-specific expression in human ontogeny where primitive and foetal liver erythroid cells that expressed high levels of γ-globin had low or absent expression of the full-length form of BCL11A (Sankaran *et al.*, 2008) but had notable expression of the shorter variant forms of the protein. Knock-down of BCL11A led to robust expression of HbF confirming its repressor function on γ-globin genes in both healthy and β-thalassaemic primary human erythroid progenitor cells (Wilber *et al.*, 2011). BCL11A was found to directly interact with chromatin at the human β-globin locus in primary human erythroid progenitor cells and physically interact with NuRD chromatin remodelling complex and erythroid transcription factors GATA1 and FOG-1 (Sankaran *et al.*, 2008). Moreover, BCL11A physically interacts with the transcription factor Sex determining region Y-box 6 (SOX6) (Xu *et al.*, 2010).

SOX6 belongs to the family of Sry-related High mobility group (HMG) box transcription factors and was shown to have a potential role in haemoglobin gene regulation. SOX6 is widely expressed in a range of tissues. The lack of activating or repressive transcriptional regulatory domains in the SOX6 protein, allows the factor to be used in multiple tissues at variable developmental stages by interacting with different partners. SOX6 plays a critical role in cell cycle progression of lineage specific progenitors as well as control the terminal differentiation of post-mitotic cells (Hagiwara, 2011). *Sox6* deficient mice demonstrated elevated embryonic β-like globin at the foetal liver stage that was rapidly down-regulated in late foetal livers (Yi *et al.*, 2006). SOX6 was shown to bind chromatin at the proximal γ-globin promoters and have long-range interactions with a variety of regions throughout the β-globin gene cluster (Xu *et al.*, 2010), suggesting a role in γ-globin gene silencing. However, SOX6 has been suggested to enhance definitive erythropoiesis in mouse by stimulating erythroid cell survival, proliferation and terminal maturation (Dumitriu *et al.*, 2006).

Direct repeat erythroid-definitive (DRED) complex was initially identified as a definitive-stage embryonic globin gene repressor with strong affinity for direct repeat

elements (DR) in the human ε-globin gene promoter (Tanimoto *et al.*, 2000). DR elements are found in both the embryonic and foetal globin gene promoters but are absent in the adult globin gene promoters. The DRED complex is composed of a heterodimer of the orphan nuclear receptors TR2 and TR4, which bind to the DR elements in the ε-globin promoter with higher affinity than the single DR element in each of the γ-globin promoters (Tanabe *et al.*, 2002). Silencing of the embryonic and foetal globin genes was delayed in definitive erythroid cells of *Tr2-Tr4*-null mutant mice suggesting a direct repression of embryonic and foetal globin gene expression in definitive erythroid cells (Tanabe *et al.*, 2007a). In addition, lysine-specific histone demethylase 1 (LSD1) and DNA methyltransferase 1 (DNMT1) act as cofactors to form the tetrameric core of DRED complex, which in turn can interact with HDAC 1 & 2, NuRD, Corepressor of element-1-silencing transcription factor (CoREST), Tripartite motif-containing 28 (TRIM28) and HDAC3 (Cui *et al.*, 2011). A point mutation at -117 of the *HBG1* ($^{A}\gamma$) promoter disrupts the DR element leading to reduced TR2/TR4 binding and increased HbF expression (Tanabe *et al.*, 2002). TR2 and TR4 can also bind to the DR element within the GATA1 haematopoietic enhancer which leads to repression of GATA1 transcription, proposing a mechanism of TR2/TR4-mediated silencing of GATA1 during terminal erythroid maturation (Tanabe *et al.*, 2007b).

Genetic association analysis led to the identification of an intergenic region between *HBS1L* and *MYB* genes on chromosome 6q23 as a quantitative trait locus (QTL) controlling foetal globin gene expression in adults (Thein *et al.*, 2007). A follow-up study proposed that this intergenic region contains an erythroid-specific enhancer that acts as a major regulatory element controlling the expression of *HBS1L* and *MYB* genes (Wahlberg *et al.*, 2009). HBS1L was excluded from having a role in regulating *HBG* (γ-globin) expression when a patient with complete loss of function of the *HBS1L* gene had a normal distribution of haemoglobin subtypes (Sankaran *et al.*, 2013). Stadhouders *et al.* (2012) showed the presence of binding sites for GATA1, LDB1, TAL1 and KLF1 within the *HSB1L/MYB* intergenic region and in close proximity to the *MYB* gene promoter. Reduction of LDB1 levels in erythroid progenitors resulted in a decrease in *Myb* promoter-enhancer interaction and transcription without affecting the transcriptional levels of *Hbs1l* (Stadhouders *et al.*, 2012, Stadhouders *et al.*, 2014). The *Myb* gene encodes a transcription factor that is critical for haematopoietic progenitors but decreases as erythroid cells mature (Emambokus *et al.*, 2003). Suzuki *et al.* (2013) demonstrated that *Hbs1l-Myb* intergenic region was responsible for the persistent

expression of embryonic globins and that despite the reduced expression of both *Hsb1l* and *Myb* genes, knock-out of *Myb* resulted in elevated expression of embryonic globin genes. *Myb* can also activate the DRED complex (Suzuki *et al.*, 2013), a repressor of embryonic and foetal globin genes in adults (Tanabe *et al.*, 2002) suggesting an indirect regulation of γ-globin transcription by this gene. Some patients with trisomy 13 have a delayed foetal-to-adult haemoglobin switch and persistence of foetal haemoglobin (Sankaran *et al.*, 2011). Sankaran *et al.* (2011) showed that the extra copy of genes (present in chromosome 13) encoding miR-15a/16-1, mediated the down-regulation of MYB transcription factor that was responsible for the increased HbF in these patients.

Among the ubiquitous *trans*-acting transcription factors, Sp1, is a zinc-finger protein belonging to the Kruppel-like factors known as Sp1/XKLF transcription factor family (Bouwman and Philipsen, 2002). Sp1 binds to the consensus sequences of GC and GT boxes in ubiquitously expressed proximal promoters, including the β-globin gene promoter, as well as to distal enhancers of housekeeping genes (Mastrangelo *et al.*, 1991). Feng and Kan (2005) suggested that binding of Sp1 to the GC and GT boxes results in the recruitment of HDAC1 that deacetylates histones and keeps the chromatin structure closed thus preventing recruitment of histone acetyltransferases (HAT) by FKLF2. Phosphorylation of Sp1 at threonine 579 results in inactivation of Sp1 and β-globin gene expression (Feng and Kan, 2005). Sp1 has been suggested to be a repressor of γ-globin expression due to the increased binding of Sp1 to the stage selector element in the human γ-globin gene promoters upon site-specific cytosine methylation (Jane *et al.*, 1993, Sengupta *et al.*, 1994).

NRF2 is a redox-sensitive transcription factor that plays a major role in the cellular defence against oxidative stress through up-regulation of transcription of phase II defence enzymes and antioxidant stress proteins. NRF2 binds to a specific ARE sequence in the promoter of target genes such as NADPH quinone oxyreductase 1 (*NQO1*) and haem oxygenase 1 (*HO1/HMOX1*). Under normoxic conditions, NRF2 is ubiquinated by KEAP1 resulting in proteosomal degradation of NRF2. In the presence of oxidative stress, KEAP1 is altered chemically by reactive oxygen species, releasing NRF2 from the complex leading to the translocation of NRF2 to the nucleus and subsequent activation of antioxidant genes (Macari and Lowrey, 2011). NRF2 is highly enriched in human embryonic stem cells and dramatically decreases upon differentiation (Jang *et al.*, 2014).

HMOX1 gene encodes haem oxygenase-1, an antioxidant enzyme that breaks down the porphyrin ring of the haem to produce biliverdin, free iron and carbon monoxide and plays a vital role in the cytoprotection of tissues during oxidative stress by preventing apoptosis (Abraham *et al.*, 2003, Brouard *et al.*, 2000). Gil *et al.* (2013) have shown that a polymorphism in *HMOX1* is associated with high HbF expression in Brazilian sickle cell patients. The authors hypothesise that reduced HMOX1 levels lead to stress-induced erythropoiesis due to higher levels of free haem (Gil *et al.*, 2013). This, however, is not proven.

The Cyclic AMP response element binding protein (CREB) is a protein belonging to basic leucine zipper domain (bZIP) superfamily of transcription factors and is highly important for transcription of haematopoietic genes and development of the myeloid lineage. Phosphorylation of CREB1 at Ser133 leads to its binding to the cAMP response element (CRE), mediating activation of transcription (Shaywitz and Greenberg, 1999). CREB1 recruits CBP which acts as a bridge by recruiting the basal transcriptional machinery to the promoters of CREB target genes (Nakajima *et al.*, 1997). CBP is important for haematopoiesis since disruption of *Cbp* in mice leads to reduced primitive and definitive haematopoiesis (Oike *et al.*, 1999). p300, a paralogue of CBP, is important for erythropoiesis since mutations at the CREB-interacting domain (KIX) result in severe defects in erythropoiesis due to disruption of the c-Myb and CREB binding sites (Kasper *et al.*, 2002). CBP stimulates *c-MYb*, *GATA1*, *NF-E2* and *KLF1* through direct interaction. Moreover, Ramakrishnan and Pace (2011) demonstrated that knock-down of *p38 MAPK* and *CREB* in primary human erythroid cultures resulted in reduced γ-globin expression while enhanced expression of both resulted in increased levels of γ-globin expression (Ramakrishnan and Pace, 2011).

1.3. Epigenetic regulators

DNA methylation was the first described epigenetic mechanism that affects gene regulation. DNA methylation involved the addition of methyl groups at the 5 position of cytosine within CpG dinucleotides by either maintenance methylation, the addition of a methyl group by DNMT1 to the symmetric CpG on the unmethylated strand of DNA after DNA replication, or by *de novo* methylation, which symmetrically methylates cytosines in dinucleotides on both strands of unmethylated DNA by DNMT3A and B

(Okano *et al.*, 1998). Globin genes of vertebrates were among the first genes to show an inverse relationship between cytosine methylation and transcription (van der Ploeg and Flavell, 1980). Methylation was therefore rendered an important factor in the regulation of γ-globin expression. In fact, studies on transgenic mice have demonstrated that DNA methylation repressed ^{A}γ-globin expression (Rupon *et al.*, 2006, Goren *et al.*, 2006). A correlation between the level of DNA methylation of five CpG residues within the γ-globin gene promoter and the level of γ-globin gene expression was established by Chin *et al.* (2009). CpG dinucleotides within the 5' γ-globin gene are unmethylated in foetal liver when the genes are expressed but methylated in adult bone marrow in both human (Mavilio *et al.*, 1983) and baboons (van der Ploeg and Flavell, 1980) when the genes are silenced.

The presence of methyl groups generates binding sites for methyl-CpG-binding domain proteins (MBDs) that consequently lead to the recruitment of histone modifying enzyme-containing co-repressor complexes. Recruitment of these complexes results in the modification of histone and consequently chromatin structure, and prevents binding of transcription factors (Goren *et al.*, 2006). Among the MBD proteins, MBD2 binds preferentially to DNA containing a high density of methylated CpGs. Knock-down of *Mbd2* expression resulted in a large increase in the expression of the silent human γ-globin gene in human β-globin locus-bearing transgenic mice (Rupon *et al.*, 2006) and in primary human CD34^{+} precursor-derived adult erythroid cells (Gnanapragasam *et al.*, 2011). MBD2 was shown to mediate silencing by recruiting the NuRD complex to methylated DNA (Zhang *et al.*, 1999, Feng and Zhang, 2001) by interacting with p66α/β, a component of the NuRD complex (Gnanapragasam *et al.*, 2011). Although MBD3 does not bind to methylated or non-methylated DNA with high affinity, it can interact with the NuRD complex which consequently interacts with the γ-globin gene promoter through association with the GATA1-associated protein FOG-1 (Miccio and Blobel, 2010). More recently, knock-down of Mi2β component of the NuRD complex showed a 10-fold increase in γ-globin gene expression in primary human erythroid cultures but was not associated with MBD2-NuRD or MBD3-NuRD interactions (Amaya *et al.*, 2013). Rather, the increase in γ-globin gene expression was partly due to down-regulation of BCL11A and KLF1 in *Mi2β* knock-down erythroid cells.

Histone acetylation was the first described histone modification (Phillips, 1963) that was associated with actively transcribed genes (Allfrey *et al.*, 1964). Acetylation of

lysine at histone tails results in charge neutralization and loosening of the interaction of nucleosomes with their associated DNA, linker DNA or adjacent histones and thus increasing accessibility of DNA to the transcriptional machinery (Zentner and Henikoff, 2013). Histone acetylation can also occur at DNA double-strand breaks and thus increasing DNA access to repair mechanisms (Xu and Price, 2011). Lysine residues are acetylated by HATs such as p300/CBP (Bannister and Kouzarides, 1996), PCAF (p300/CBP-associated factor) and TAF(11)250 (TBP associated factor) (Mizzen *et al.*, 1996), which usually have low substrate specificity. Lysine deacetylation is performed by histone deacetylases (Kuo and Allis, 1998). Analysis of mononucleosomes from primitive and definitive erythrocytes in chicken embryos showed that both active and inactive genes were hyperacetylated suggesting a broad acetylation pattern of chromatin domains (Forsberg and Bresnick, 2001). In addition, hyperacetylated areas were associated with DNaseI sensitivity (Hebbes *et al.*, 1994) suggesting that locus-wide hyperacetylation promotes the chicken β-globin locus for transcription but does not control β-globin gene switching. A broad hyperacetylation pattern was also observed in the murine β-globin locus, however, without being uniform. Hyperacetylation was concentrated on Histone 3 (H3) and Histone 4 (H4) at the LCR and the active β-major and β-minor promoters in expressing but not in non-expressing tissues, with a hypoacetylated subdomain encompassing the silenced embryonic genes (Forsberg *et al.*, 2000). Restoration of acetylation by using HDAC inhibitors was not sufficient to reactivate the silenced embryonic genes suggesting that requisite factors are missing in the definitive cells. Analysis showed the presence of a basal H3 and H4 acetylation throughout the human β-globin locus in MEL cells, with peaks of H3 acetylation at the LCR and the active promoters. Deletion of HS2-4 abolished expression of β-like globin genes but maintained the ability to form the remaining HS and an open chromatin conformation suggesting that basal H3 and H4 acetylation is correlated with DNaseI sensitivity (Reik *et al.*, 1998). Based on the above observations, it can be proposed that the broad acetylation pattern of the β-globin locus in erythroid cells is necessary for transcriptional competence of the β-globin genes where other types of regulation would then determine whether a single gene is transcribed or not (Bresnick and Tze, 1997). Experimental evidence demonstrated that developmentally regulated transcription of intergenic regions of εγ and δβ domains has the capacity to increase chromatin structure accessibility above the basal state, rendering it an essential component of the remodelling process of chromatin domains (Gribnau *et al.*, 2000).

In contrast to DNA methylation, methylation of histones at lysine residues can be associated with gene activation, gene silencing or a bivalent function. In particular, methylation of histone H3 Lysine 4 (H3K4) and histone H3 lysine 36 (H3K36) is associated with open chromatin and gene transcription, while histone H3 lysine 9 (H3K9) and histone H3 lysine 27 (H3K27) methylation are involved in transcriptional repression and gene silencing. Histone methylation provides recognition sites for the recruitment of non-histone proteins and enzymes which affect downstream gene functions (Kiefer *et al.*, 2008) and increase the binding affinity of many domains including Tudor, chromodomains, and Plant Homodomain (PHD) (Taverna *et al.*, 2007). Among the major writers of histone methylation is protein arginine methyltransferase 5 (PRMT5) which was shown to be mechanistically linked to silencing of γ-globin gene (Zhao *et al.*, 2009). In addition, PRMT1, another member of the PRMT family, has been associated with human γ-globin gene silencing through association with FoP. Interestingly, PRMT1 promotes acetylation of Lys9/Lys14 by recruitment of dimethyl Histone 4 arginine 3 (H4R3) to PCAF and subsequent transcription of the adult β-globin gene (Li *et al.*, 2010) suggesting that PRMT1 can silence γ-globin gene expression by increasing interactions between the LCR and the β-globin gene. Specific lysine demethylases (LSD) were also shown to be involved with γ-globin gene silencing in both human and murine erythroid cells. LSD1 has been shown to associate with the transcription factor BCL11A through a complex containing the CoREST (Xu *et al.*, 2013). Inhibition of LSD1 by RNAi or by the monoamine oxidase inhibitor tranylcypromine in human erythroid cells or β-globin–transgenic mice enhances γ-globin expression (Shi *et al.*, 2013).

The ability of DNA methyltransferase inhibitor drugs such as decitabine, to cause secondary histone modifications in addition to their primary epigenetic role, proposes that epigenetic regulation of globin gene expression is mediated by the interplay between transcription factors and coregulatory complexes that contain mediators for epigenetic chromatin changes. BCL11A, a dominant repressor of γ-globin gene in primary human erythroid progenitor cells, is shown to interact with the MBD3-NuRD complex, the LSD1/CoREST complex, Nuclear receptor corepressor/ Silencing mediator of retinoid and thyroid hormone receptors complex (NCoR/SMRT) and DNMT1 (Xu *et al.*, 2013). In addition, the TR2/TR4/DRED orphan receptor complex was also shown to interact with the above complexes (Cui *et al.*, 2011). Epigenetic regulators could also be involved in γ-globin gene silencing through epigenetic

regulation of the expression of the transcription factors themselves. One such example is the function of Mi2β/CHD4 (Chromodomain helicase-DNA-binding protein 4) in the regulation of KLF1 and BCL11A expression in primary human erythroid cells independently from NuRD complex (Amaya *et al.*, 2013).

1.4. Thalassaemias

Thalassaemias are characterised by a reduced rate of synthesis of structurally normal globin chains and result from mutations in the globin genes. There are two major types of thalassaemias, α and β, which result from reduced or absent synthesis of α- or β-globin chains, respectively. α- or β-thalassaemias are prevalent in areas where *Plasmodium Falciparum* malaria is or has been endemic due to a natural protection of heterozygote thalassaemias against the Falciparum malaria parasite (Weatherall, 1987, Allen *et al.*, 1997, Weatherall, 2008). However, as a result of migration, thalassaemias are now the commonest inherited disorders worldwide with at least 60000 severely affected individuals born every year (Modell and Darlison, 2008). Although malaria eradication programmes in developing countries such as Cyprus have reduced the frequency of thalassaemias, it will probably take many generations before any significant change in the allele frequency occurs (Weatherall, 2010, Kyrri *et al*., 2013). Therefore, due to the economic burden of treating thalassaemic patients, developing countries are now reducing the incidence of β-thalassaemia by incorporating prenatal diagnosis and genetic counselling in their health plan (Cao *et al.*, 1990).

Other types of thalassaemias include rarer forms of the disease in which other β-like globin genes (or a combination of different globin genes) are affected (Weatherall and Clegg, 2001). α- and β-thalassaemias can be further sub-classified based on the variable effect of each mutation on globin chain synthesis. Thalassaemias are inherited in a Mendelian recessive manner and are very heterogeneous in the clinical picture they produce, ranging from very severe anaemias to asymptomatic conditions (Weatherall and Clegg, 2001).

1.4.1. Alpha-thalassaemia

Alpha-thalassaemias mainly arise from deletions of the α-globin gene resulting in whole or part of the α-globin gene missing. Occasionally point mutations in critical regions of the α-globin genes give rise to the non-deletional α-thalassaemia variants which lead to inactivation of the α-globin genes (Higgs, 2013). The normal α-globin genotype is αα/αα with two α-globin genes in each chromosome (α_2 and α_1) being inherited from each parent. Therefore, deletion or inactivation of all four α-globin genes abolishes α-globin synthesis and results in the failure to produce foetal haemoglobin leading to severe anaemia and death in the uterus, a condition known as hydrop foetalis (Chui *et al.*, 2003). If, however, three of the four α-globin genes are deleted or inactivated then this leads to severe to moderate anaemia in adulthood and the production of Hb Bart's (γ4) in foetal life (Lorey *et al.*, 2001, Higgs, 2013) and HbH (β4) in adult life, hence the term HbH disease (Chui *et al.*, 2003, Higgs, 2013). HbH disease results in moderately severe microcytic, hypochromic anaemia with splenomegaly and jaundice (Origa *et al.*, 2007). Individuals with two defective α-globin genes (α-thalassaemic trait) may present with mild anaemia associated with hypochromic microcytic red blood cells, while individuals with one defective α-globin gene have few recognisable changes in their haematology (Vichinsky, 2013). There are approximately 222 different molecular defects currently known to cause α-thalassaemia and the clinical phenotype and severity of each defect depend on the degree of deficiency in α-globin chain production (Harteveld and Higgs, 2010, Kountouris *et al.*, 2014). Deletions leading to a complete absence of alpha globin chain synthesis are known as α^0-thalassaemia, while deletions that only reduce the synthesis of normal alpha globin chains are known as α^+-thalassaemia (Higgs, 2013).

1.4.2. Beta-thalassaemia

β-thalassaemia, in contrast to α-thalassaemia, is mainly caused by point mutations in the β-globin gene or its immediate flanking regions and rarely due to deletions (Weatherall and Clegg, 2001). More than 300 mutations have been identified so far for β-thalassaemia, the majority of which are point mutations (Kountouris *et al.*, 2014). Point mutations include mutations within the promoter and 5' UTR leading to defective β-globin gene transcription, mutations within splice-junctions, consensus sequences and

3’ UTR affecting messenger RNA processing; and nonsense, frameshift and initiation codon mutations resulting in abnormal mRNA translation (Olivieri, 1999, Cao and Galanello, 2010).

Thalassaemia major, the most severe end of the disease spectrum, is characterised by severe reduction or complete absence of β-globin production due to the inheritance of two severe β-thalassaemic alleles in a homozygous or compound heterozygous state. The lack of β-globin chains leads to an imbalanced globin chain synthesis and accumulation of excess unbound α-globin chains. The excess α-globin chains precipitate in erythroid precursor cells causing membrane damage and premature destruction of erythroid precursors in the bone marrow and at extramedullary sites (Thein, 2002), a phenomenon termed ineffective erythropoiesis. Anaemia caused by ineffective erythropoiesis in the bone marrow and haemolysis in peripheral blood promotes bone marrow expansion to compensate for the reduced erythrocytes (Thein, 2004). In addition to the huge numbers of erythroid precursors, the bone marrow of a thalassaemic patient holds fifteen times the number of apoptotic cells at the polychromatic and orthochromatic stages of maturation compared to a healthy individual (Centis *et al.*, 2000).

Thalassaemia major patients present with a severe anaemia, which only becomes apparent at 3-6 months after birth when the switch from foetal to adult haemoglobin is almost complete and the predominant Hb is adult haemoglobin (Olivieri, 1999). Failure to transfuse these children, leads to death of the child within the first decade of life. β-thalassaemia is a progressively debilitating disease which can involve life-long damage to multiple organs. Patients appear with hepatospenomegaly due to excessive red cell destruction, extramedullary haematopoiesis and iron overload (Thein, 2004). Iron overload is one of the major causes of mortality and morbidity associated with β-thalasseamia and occurs partly from chronic blood transfusion (transfusional haemosiderosis) and partly due to increased intestinal iron absorption secondary to inhibition of hepcidin synthesis (Atanasiu *et al.*, 2007). Iron deposition in visceral organs such as the heart, liver and endocrine glands, leads to tissue damage and ultimately organ dysfunction and failure (Rund and Rachmilewitz, 2005). Myocardial disease caused by transfusional siderosis is the most common life-limiting complication of iron overload in β-thalassaemia patients with a mortality rate of 67% (Borgna-Pignatti *et al.*, 2004, Cao and Galanello, 2010).

Individuals with milder anaemias that do not require regular transfusions have thalassaemia intermedia or non-transfusion dependent thalassaemia (NTDT). Thalassaemia intermedia comprises of a clinically and genotypically very heterogeneous group of thalassaemia-like disorders, ranging in severity from mild anaemias which are more severe than the asymptomatic carrier (thalassaemia minor) to delayed severe anaemia that leads to blood transfusions (Forget and Bunn, 2013). Patients carry homozygous or compound heterozygous alleles for β-thalassaemia and generally present with anaemia later in life compared to those with thalassaemia major. In individuals with two severe beta-thalassaemic alleles, the molecular basis for NTDT, include co-inheritance of α-thalassaemia that reduces the α-globin chain imbalance and co-inheritance of mutations associated with increased production of foetal (γ) globin chains in adults as the γ-globin combine with the excess α-globin chains to form functional foetal haemoglobin molecules (Weatherall and Clegg, 2001).

Individuals with a single β-thalassaemia mutation are known as carriers. The clinical phenotype of β-thalassaemia carriers (minor) is usually asymptomatic characterised by hypochromic, microcytic blood picture with mild or no anaemia and an increase in HbA_2 (Forget and Bunn, 2013).

1.5. Conventional and curative therapies

The only extensively used approach in managing β-thalassaemia is regular blood transfusions. Blood transfusions lead to iron overload and hence are usually accompanied by iron chelation therapy. Iron chelation therapy was found to double the life expectancy of patients with thalassaemia major (Olivieri, 1999). There are currently three available drugs used for iron chelation therapy. Deferoxamine, the most commonly used iron chelator, is administered as parenteral infusions (Brittenham *et al.*, 1994). Deferiprone (Aydinok *et al.*, 2007) and deferasirox (Piga *et al.*, 2006, Cappellini *et al.*, 2006), are oral iron-chelators. Blood transfusions and chelation therapy need to be carried out throughout the life of the patient.

While conventional treatment has the capacity to improve the quality of life of people with thalassaemia major, allogeneic haematopoietic stem cell transplantation is at the moment the only curative treatment for the disease. Since 1982 when the first successful

transplantation was performed (Thomas *et al.*, 1982), haematopoietic stem-cell transplantation has greatly advanced as a treatment for thalassaemia major, with enormous progress made in the areas of donor identification and selection and development of alternative sources of haematopoietic stem cells (Rund and Rachmilewitz, 2005). The donor pool has been increased by the use of matched unrelated or haploidentical donors and using cord blood HSCs (Pinto and Roberts, 2008). More recently, the use of peripheral blood as a source of HSCs stem cells has replaced the use of bone marrow for a significant proportion of allogeneic transplantations (Mahmoud *et al.*, 1999, Tan *et al.*, 2004). Therefore, haematopoietic stem-cell transplantation has now become a more viable approach for the treatment of thalassaemia. However, stem-cell transplantation is only available to those with access to high-technology medicine and not to all the individuals with β-thalassaemia.

Gene therapy, which corrects the defective β-globin gene or introduces a functional β-globin gene in autologous HSCs, is an attractive potential therapeutic alternative for people with β-thalassaemia (Sadelain *et al.*, 2005). In principle, haematopoietic stem cells from the patient are harvested and infected with modified viruses containing the modified β-globin gene and its upstream regulatory elements. These autologous stem cells are then inserted back into the patient after myeloablation to create a niche for the corrected progenitors. Although this will eliminate the problem of compatible donor availability, limitations of the method arise in vector construction (Arumugam and Malik, 2010), including the safety of the vector in terms of recombination or mutagenesis and also the ability of the vector to accommodate all the sequences required for correct expression of the β-globin gene (Puthenveetil and Malik, 2004). Current investigations are focusing on vector production methodologies and safety issues aiming to minimise insertional oncogenesis (Nienhuis, 2008). A number of clinical trials are currently taking place to test lentiviral constructs for treatment of β-thalassaemia.

One approach to overcome the integration problems caused by viral vectors is the use of non-integrating gene editing to correct disease-causing mutations in HSC. Engineered nucleases composed of sequence-specific DNA-binding domains fused to a non-specific DNA cleavage module, induce targeted DNA double-strand breaks (DSBs) that subsequently stimulate the cellular DNA repair mechanisms, including error-prone non-homologous end joining (NHEJ) and homology-directed repair (HDR) (Wyman and

Kanaar, 2006) to correct the mutation. Three different genome editing systems have been introduced; zinc finger nucleases (ZFNs) (Urnov *et al.*, 2005), transcription activator-like effector nucleases (TALENS) (Miller *et al.*, 2011) and RNA-directed Cas9 nucleases (Cho *et al.*, 2013, Mali *et al.*, 2013). In contrast to ZFNs, TALENs can be easily engineered to specifically recognise any given DNA sequence with high efficiency (Hockemeyer *et al.*, 2011), lower off-target effects and reduced nuclease-associated cytotoxicities (Mussolino *et al.*, 2011). Recent studies reported the successful correction of *HBB* mutations in β-thalassaemia and sickle cell anaemia using TALENs (Ma *et al.*, 2013, Sun and Zhao, 2013). However, the efficiency of TALENS can be hampered by DNA methylation and histone acetylation in active chromatin. In contrast, Cas9 nucleases are not limited by these obstacles and can be easily manipulated (Deltcheva *et al.*, 2011). Xie *et al.* (2014a) efficiently corrected two different β-thalassaemia mutations and converted homozygous thalassaemia to the heterozygous states in patient-derived iPS cells by the combinational use of CRISP/Cas9 for *HBB* cleavage and *piggyback* for both positive and negative selection of corrected clones. The *piggyback* transposon is a mobile genetic element that contains a bi-functional fusion protein between puromycin N-acetyltransferase and a truncated version of herpes simple virus type 1 thymidine kinase (Chen and Bradley, 2000). Two 500bp segments of the genomic sequences of *HBB* intron 1 were incorporated into *piggyback* transposon which upon recognition allow the transposon to be integrate into the chromosome via a 'cut and paste' mechanism. Puromycin resistance was used for positive selection of clones with homologous recombination events. Upon transient transfection of transposase, *piggyback* cassette was removed from the mutation-corrected clones and negative selection using thymidine kinase eliminated the cells that retained the cassette.

Stem cell research was introduced recently as an upcoming therapeutic strategy due to the discovery that human somatic cells can be reprogrammed to form embryonic-like multipotent cells by the introduction and expression of four transcription factors (Oct4, Sox2, KLF4 and c-Myc) (Takahashi and Yamanaka, 2006). The introduction of iPS cells has created opportunities for their exploitation as new human disease models, new methods for drug screening while offering the potential for new approaches to cell therapy. The proof of suitability of such a system for thalassaemia came from a study which generated iPS cells from somatic cells of a sickle cell mouse (Hanna *et al.*, 2007). Similarly, reprogrammed human skin fibroblasts from a patient with homozygous β-thalassaemia into iPS cells (Ye *et al.*, 2009) had the ability to differentiate successfully

into red blood cells (Chang *et al.*, 2011). Although iPS cells have opened a whole new approach into therapeutic approaches for haemoglobinopathies, extensive research needs to be done in order to overcome major challenges in this field before iPS cells can be considered a clinical therapeutic option.

Epidemiological data have shown that co-existence of hereditary persistence of foetal haemoglobin (HPFH) in patients with β-thalassaemia ameliorates the severity of the β-thalassaemia phenotype. HPFH is characterised by significantly high foetal haemoglobin production in adulthood due to deletions in the β-globin gene cluster or point mutations in the γ-globin gene promoters that lead to deregulation of the normal developmental regulation of γ-globin gene expression. Genetic studies have shown that the excess γ-globin chains due to HPFH, associate with the excess free α-globin chains accumulating due to β-thalassaemia thus increasing the synthesis of fully functional haemoglobin (foetal) and reducing the accumulation of free α-globin chains and subsequently, ameliorating the clinical symptoms of β-thalassaemia. Reactivation of foetal haemoglobin is therefore considered to be another therapeutic approach for β-thalassaemia. Pharmacological therapy and gene therapy are the two methods currently being investigated for induction of foetal haemoglobin. Gene therapy involves the use of gene editing to target globin suppressors such as *BCL11A* or through transduction of γ-globin genes. While gene therapy is still at experimental stage, different classes of pharmacological agents able to reactivate HbF synthesis have been identified both *in vitro* and *in vivo*. Despite the demonstration of notworthy results for some of the agents in clinical trials, many of these agents have low efficacy and specificity while others are potentially toxic or carcinogenic (Gambari and Fibach, 2007).

1.6. Pharmacological reactivation of foetal haemoglobin

Several studies have focused on the identification of potential HbF inducers. More than 70 agents (Appendix I) have been identified so far and can be grouped in several classes according to their chemical structure and molecular mechanism.

A correlation between globin gene expression and DNA hypomethylation in human erythroblasts purified at various stages of differentiation (Mavilio *et al.*, 1983) led to the introduction of 5-azacytidine as a potential HbF inducer. Analysis of the β- and γ-globin

gene promoters has shown that the γ-globin promoter was hypomethylated in foetal erythroid progenitors and was becoming progressively hypermethylated as erythroid differentiation progressed into adult erythroid progenitors (Singh *et al.*, 2007). Similarly, the γ-globin gene promoter in $CD34^+$ cells was hypomethylated at the pre-erythroid phase and became hypermethylated at later stages of differentiation (Mabaera *et al.*, 2007). Induction of γ-globin gene activation by 5-azacytidine, a DNMT inhibitor, was correlated with hypomethylation of a specific CpG site located 53-bp upstream of the γ-globin transcription initiation site (Ley *et al.*, 1983). However, it was proposed that the inducing activity of the agent might be partly due to its ability to act as an S-phase cell-cycle inhibitor rather than as a DNA hypomethylator. As a result, studies started investigating the use of S-phase inhibitors as potential HbF inducers. Among the cytostatic agents tested, hydroxyurea was identified as a highly effective HbF inducer. Investigation of hydroxyurea in small clinical trials demonstrated a robust HbF inducing response in patients with sickle cell anaemia (Platt *et al.*, 1984). Further investigation of the use of hydroxyurea in larger clinical trails (Charache *et al.*, 1995) led to FDA approval for its use in patients with sickle cell disease. In the meantime, the observations that infants born to diabetic mothers had a delayed foetal to adult haemoglobin switch led to suggestions that elevated hydroxybutyrate levels in utero could be responsible for the elevated HbF levels (Perrine *et al.*, 1985, Bard and Prosmanne, 1985). These observations let to the introduction of butyrate and short chain fatty acids (SCFA) as potential HbF inducers. Despite promising results with these agents in initial studies (Perrine *et al.*, 1994), the effect of the agents in larger clinical trials did not produce a wide response in all patients (Sher *et al.*, 1995). It was therefore thought that SCFA induce HbF through the inhibition of HDAC (Fathallah *et al.*, 2007), leading to the study of a variety of HDAC inhibitors *in vitro* and *in vivo* for their clinical efficancy as HbF inducers (Cao, 2004). Later studies demonstrated the ability of DNA binding (Bianchi *et al.*, 1999) and immunomodulatory drugs (Grzasko *et al.*, 2006, List *et al.*, 2006) as potential HbF inducers. While trials utilizing all the above agents have proven that pharmacological activation of foetal haemoglobin can be an effective therapeutic approach, none of these agents are optimal inducers in a clinical setting due to their short and long-term side effects and convenience of administration.

Attempts to identify novel HbF inducers are based on the primary actions of specific classes of inducing agents such as global DNA hypomethylation by DNA methyltransferase inhibitors and global histone hyperacetylation by HDAC inhibitors.

However, none of these models can explain the ability of such a functionally diverse group of agents to induce HbF levels. As a result, major focus has been directed to investigate the role of cell signalling in the reactivation of HbF by these agents. In the meantime, while most of the research on HbF induction has been focused on the increase of γ-globin gene expression, experimental evidence suggested that post-transcriptional processes have a role in the mechanism of HbF induction (Weinberg *et al.*, 2005, Mabaera *et al.*, 2008a).

1.6.1. 5-azacytidine and decitabine

5-azacytidine was the first agent discovered to reactivate HbF synthesis in humans. 5-azacytidine was originally brought to the clinic as a cytotoxic cancer chemotherapy agent but it was later shown to be a potent DNA methyltransferase inhibitor (Christman, 2002). DeSimone *et al.* (1982b) showed that 5-azacytidine increased HbF levels from 6 to 30 times higher than those produced by bleeding alone. However, the exact mechanism of action of this agent is unclear as it acts both as a cytotoxic anticancer chemotherapy agent and as an inhibitor of DNA methyltransferase enzyme (Testa, 2009). Due to its multiple effects, two theories have been proposed to explain the HbF inducing action of 5-azacytidine. The first theory suggests that the cytotoxic effect of the agent on erythroid progenitors stimulates a compensatory erythroid cell production due to erythropoietic stress, leading to an increase in HbF synthesis (Stamatoyannopoulos *et al.*, 1985). The second theory involves the agent's DNA methyltransferase inhibitory activity and suggests that demethylation of the γ-globin gene promoter leads to consequent transcriptional de-repression of the γ-globin gene (DeSimone *et al.*, 1982b). More recent studies indicate that neither theory is correct as small interfering RNA silencing DNA methyltransferases failed to induce HbF synthesis. Moreover, the same studies also demonstrated no changes in growth or differentiation kinetics in the presence of 5-azacytidine suggesting an unknown mechanism of action (Mabaera *et al*., 2008a).

Subsequent clinical trials of 5-azacytidine in sickle cell and thalassaemia patients have confirmed its ability to increase HbF synthesis and reduce anaemia (Charache *et al.*, 1983, Ley *et al.*, 1982, Ley *et al.*, 1983). However, despite its promising effects, further trials raised concerns over its carcinogenic potential in humans (Carr *et al.*, 1984). A

safer derivative of 5-azacytidine, decitabine (5-aza-2'-deoxycytidine), was introduced that was able to increase HbF synthesis in both baboons and sickle cell patients at 10-20% the concentration of 5-azacytidine (Koshy *et al.*, 2000, Saunthararajah *et al.*, 2003, Saunthararajah *et al.*, 2008). Decitabine is a hypomethylating agent which depletes the chromatin modifying enzyme DNMT1 and thus hypomethylates DNA CpG nucleotides. *In vitro* experiments showed that decitabine favours erythroid and megakaryocytic differentiation of haematopoietic cells (Visvader and Adams, 1993). Decitabine was shown to increase acetylation of H3 and H4 at the γ-globin promoter specifically suggesting that decitabine-mediated changes in DNA methylation might be associated with changes in chromatin structure (Saunthararajah *et al.*, 2003). However, hypomethylation of the ε-promoter did not lead to increased expression of the gene, suggesting that local hypomethylation is not the sole requirement for gene expression (Karpf *et al.*, 2004).

1.6.2. Butyrate and short chain fatty acids

Ginder *et al.* (1985) was the first to demonstrate that administration of butyrate resulted in induction of embryonic globin gene expression in 5-azacytidine pre-treated chickens. Shortly after, infants born to diabetic mothers who were exposed to higher butyrate levels *in utero* were shown to have higher HbF levels at birth (Perrine *et al.*, 1985; Bard *et al.*, 1985). Moreover, administration of butyric acid in baboons resulted in the reversion of foetal to adult globin expression (Constantoulakis *et al.*, 1988). Despite the lack of understanding of the mechanism of action of butyrate, it was proposed that the increase in HbF levels in the presence of the agent was due to changes in histone acetylation level of promoter regions due to the function of butyrate as an inhibitor of HDAC. Subsequent studies have shown that activation of γ-globin gene by butyrate and other SCFA was dependent on a duplicated CCAAT box sequence, located upstream of the transcription initiation site (McCaffrey *et al.*, 1997). Due to its promising stimulatory effect of HbF synthesis, a pharmaceutical preparation of butyrate, arginine butyrate, was administered to patients with sickle cell disease and β-thalassaemia as infusions. Butyrate infusions were able to stimulate HbF synthesis in sickle cell patients (Perrine *et al.*, 1993) but the effect observed was not sustainable, possibly due to an inhibitory effect of the drug on haematopoiesis (Sher *et al.*, 1995). A major drawback of

the drug was the inability to stimulate HbF synthesis in all patients (Sher *et al.*, 1995). Oral alternatives of the drug resulted in enhancement of HbF synthesis in β-thalassaemia patients. Sodium phenylbutyrate promoted a response in patients with high endogenous erythropoietin levels with no related globin gene mutation (Collins *et al.*, 1995) while isobutyramide elicited a response in transfusion-dependent β-thalassaemia patients with high pretreatment HbF levels (Reich *et al.*, 2000).

Despite initial assumptions that butyrate and SCFA induce foetal haemoglobin through histone acetylation, not all SCFA caused global histone hyperacetylation (Boosalis *et al.*, 2001). Fathallah *et al.* (2007) demonstrated that while butyrate increased histone acetylation at the γ-globin promoter, it decreased histone acetylation at the β-globin promoter. These findings suggest an alternative mechanism of SCFA in the stimulation of HbF induction.

1.6.3. Hydroxyurea

Hydroxyurea (HU) is the most widely studied HbF inducer and was initially used for the treatment of myeloproliferative disorders. The mechanisms underlying HU action still remain elusive but HU has the ability to inhibit cellular ribonucleotide reductase, whose role is critical for DNA synthesis in the dividing late progenitor cells. Initial studies carried out in 1984 in anaemic monkeys (Letvin *et al.*, 1984) and in sickle cell patients (Veith *et al.*, 1985) showed stimulation of HbF production by HU. Fibach *et al.* (1993) showed that HU increased the level of HbF synthesised by erythroblasts using two-phase liquid erythroid cell cultures. In addition, it was shown that HU decreased erythroblasts proliferation and increased cell size and haemoglobin content per cell (Fibach *et al.*, 1993). Data from various studies support the hypothesis that HU is able to induce the nitric oxide (NO) dependent activation of soluble guanylyl cyclase (sGC) (Cokic *et al.*, 2008) in primary human erythroid progenitor cells and in patients with sickle cell disease (Nahavandi *et al.*, 2002). HU also increases cyclic guanosine monophospate (cGMP) and cyclic adenosine monophospate (cAMP) in primary human erythroid progenitor cells (Keefer *et al.*, 2006). Moreover, HU was shown to stimulate the secretion-associated and RAS-related (SAR) GTP-binding protein, which in turn induces apoptosis and G1/S phase arrest by reducing phosphoinositol-3 kinase (PI3K) and extracellular-signal regulated kinase (ERK) phosphorylation while increasing p21

and GATA2 expression (Tang *et al.*, 2005). Evidence that HU decreases vaso-occlusion, acute chest syndrome and transfusion requirements led to its approval as a therapeutic agent in patients with sickle cell disease by the FDA in 1998 (Charache *et al.*, 1995). A double blind randomised trial of hydroxyurea in children with sickle cell disease demonstrated that HU reduced acute chest syndrome, pain and dactylitis as well as transfusion requirements while improved haematological parameters and increased foetal haemoglobin levels (Wang *et al.*, 2011). One of the major disadvantages of HU is that not all patients respond to the agent. Around 60-80% of the patients respond to HU treatment with pre-therapy HbF levels being a good predictor of response to HU. In patients with β-thalassaemia, HU is much less effective (Italia *et al.*, 2009). Response to HU treatment was correlated with XmnI haplotypes in β-thalassaemia patients (Yavarian *et al.*, 2004, Bradai *et al.*, 2007). More positive results were reported in patients with NTDT (Ehsani *et al.*, 2009, Kosaryan *et al.*, 2014).

1.6.4. Histone deacetylase inhibitors

Histone acetylation analysis of the human β-globin locus showed that the LCR is acetylated at the same level in both foetal and adult erythroblasts while the level of acetylation in the globin gene promoters was correlated to the level of transcription (Kim and Dean, 2004). Based on these findings as well as the action of butyrate as a HDAC inhibitor, several other HDAC inhibitors including MS-275, apicidin (Witt *et al.*, 2003), scriptaid (Johnson *et al.*, 2005) and trichostatin A (Pace *et al.*, 2003) were tested and shown to induce HbF synthesis both in erythroleukaemia cell lines and primary human erythroid cultures. Computational modelling and screening of chemical libraries, identified RB7 as a potent HbF inducer in cultured erythroid progenitors, by dissociating HDAC3 from its adaptor protein or from the γ-globin gene promoter, thus promoting the recruitment of RNA polymerase II to the promoter (Mankidy *et al.*, 2006). Several studies have shown that most of these HDAC inhibitors have an indirect action on the globin gene promoter through the induction of p38 MAPK pathway (Pace *et al.*, 2003, Tamura *et al.*, 2000, Wei *et al.*, 2007) by phosphorylation of CREB1 and activating factor-2 (ATF-2) (Sangerman *et al.*, 2006). The phosphorylated transcription factors then bind to the cyclic AMP responsive element present in the promoter region of the γ-globin genes and consequently activate these genes (Sangerman *et al.*, 2006).

1.6.5. DNA-binding Drugs

DNA-binding drugs (DBD) interfere with critical cellular processes such as inhibition of DNA and RNA polymerases, topoisomerase and nucleases. Mechanistically, DBDs may block the action of enzyme complexes or interfere with protein access to DNA by inhibiting the interaction between transcription factors and target DNA elements.

Mithramycin, chromomycin, tallimustine and angelicin are some of the DBDs found to be effective in reactivating HbF synthesis. Most of these compounds have been used previously in therapy of other diseases such as cancer and hypercalcaemia (chromomycin and mithracycin), viral infections (tallimustin) and psoriasis (angelicin). Bianchi *et al.* (1999) showed that mithramycin and chomomycin were powerful inducers of erythroid differentiation in K562 cells, by increasing the content of foetal haemoglobin and increasing the γ-globin mRNA accumulation. Furthermore, mithramycin (Fibach *et al.*, 2003), tallimustin (Chiarabelli *et al.*, 2003), ciplastin (Bianchi *et al.*, 2000) and angelicin (Lampronti *et al.*, 2003) enhanced HbF synthesis in human erythroid progenitor cells. Interestingly, the level of induction displayed by mithramycin was higher and more consistent than hydroxyurea in thalassaemic erythroid progenitor cells while showing lower cytotoxicity than hydroxyurea (Fibach *et al.*, 2003). Similarly, three DNA-binding antibiotics (doxorubicin, idarubicin and daunorubicin), also known as anthracyclins, increased γ-globin expression in primary human erythroid progenitor cells from healthy and thalassaemic cultures above the effect of hydroxyurea (Spyrou *et al.*, 2010).

1.6.6. Immunomodulatory drugs

Thalidomide, originally synthesised as an anti-histaminic agent, was prescribed as a sedative during pregnancy. Due to its teratogenic effects, thalidomide was withdrawn from the market (Mcbride, 1961), but re-introduced due to is remarkable effect in erythema nodosum leprosum and was FDA approved as an anti-inflammatory drug (Nightingale, 1998). It is currently being used to treat dermatologic, infectious, autoimmune and haematologic disorder including multiple myeloma. Clinical trials have demonstrated the ability of thalidomide to improve anaemia in younger patients with low-risk myelodysplastic syndromes and to stimulate erythropoiesis in patients

with multiple myeloma (Grzasko *et al.*, 2006). Thalidomide was able to reduce transfusion-dependent anaemia in patients with myelodysplasia (Raza *et al.*, 2001). Preliminary data suggest that thalidomide increases expression of the γ-globin gene via reactive oxygen species (ROS)-dependent activation of the p38 MAPK signalling pathway and histone H4 acetylation (Aerbajinai *et al.*, 2007). However, its use as an HbF inducer is limited by its teratogenic activity. More recently, lenalidomide and pomalidomide, two thalidomide analogues, were introduced as HbF inducers. Lenalidomide and pomalidomide were described to have immunomodulatory properties as well as inhibitory activities of Tumour necrosis factor (TNF)-α production (Corral *et al.*, 1996). Similarly to thalidomide, lenalidomide, which was recently approved by the FDA as a drug for myelodysplastic syndrome (MDS), was shown to reduce and eliminate the need for red blood cell transfusion in patients with this disease (List *et al.*, 2006). Moutouh-de Parseval *et al.* (2008) demonstrated that pomalidomide and lenalidomide were able to induce HbF expression by regulating the transcription of γ- and β-globin genes in primary human erythroid progenitor cells from patients with sickle cell disease while reducing erythroid differentiation. Furthermore, these agents show no cytotoxicity to the erythroid progenitors but actually reduce the number of erythroid differentiated cells undergoing apoptosis (Verhelle *et al.*, 2007). The combination of stimulating erythropoiesis and increasing erythroid progenitor survival makes these drugs ideal candidates for the treatment of ineffective erythropoiesis observed both in MDS and haemoglobinopathies (Moutouh-de Parseval *et al.*, 2008).

1.7. The role of cell signalling in HbF induction

Initially most HbF inducing agents have been viewed as affecting γ-globin gene expression by altering the chromatin structure of globin gene promoters in the case of DNA demethylating agents and histone deacetylase inhibitors, or by altering the kinetics of erythroid differentiation in the case of cytotoxic agents. However, findings such as the ability of 5-azacytidine to increase HbF synthesis in the absence of global DNA methylation, the inability of the DMNT1 knock-down to induce expression of the γ-globin gene through hypomethylation of its promoter (Mabaera *et al.*, 2008a), the ability of drugs to induce HbF *in vitro* without changes in the differentiation kinetics (Mabaera *et al.*, 2008a), the ability of short chain fatty acids that do not affect histone

acetylation to induce HbF synthesis (Boosalis *et al.*, 2001) and the ability of p38 MAPK inhibitors to block γ-globin and HbF induction (Witt *et al.*, 2000), has raised questions about the molecular mechanism of action of these agents. In fact research into the mechanisms of action of some of the known HbF inducers has identified different signalling pathways that might be involved in γ-globin gene reactivation. The implicated pathways include cGMP and cAMP, NO, p38 MAPK, ROS and cytokine signalling. The first evidence came from Witt *et al.* (2000) who showed that induction of erythroid differentiation and haemoglobin production in K562 by butyrate was associated with increased p38 MAPK phosphorylation and that inhibition of p38 activity prevented butyrate's effect. In addition, induction of HbF by HDAC inhibitors was shown to be mediated not only by hyperacetylation but also due to production of ROS (Hsiao *et al.*, 2006) that lead to p38 MAPK phosphorylation and subsequent activation of the cAMP response element binding protein (CREBP) and ATF2 (Pace *et al.*, 2003; Sangerman *et al.*, 2006). Primary human erythroid cultures (Aerbajinai *et al.*, 2007) demonstrated that thalidomide has the ability to increase γ-globin gene expression through ROS production, p38 MAPK phosphorylation and global enhancement of histone H4 acetylation. Moreover, Ikuta *et al.* (1998) demonstrated the importance of cGMP/sGC pathway activation in the butyrate mediated induction of γ-globin gene expression. However, cAMP signalling has greater importance in HbF induction than cGMP in *in vitro* primary human erythroid experiments (Keefer *et al.*, 2006). Bailey *et al.* (2007) reported that pharmacological manipulations that increased cAMP stimulate HbF synthesis in primary human erythroid progenitor cells from β-thalassaemia intermedia patients. In addition, intracellular levels of cAMP and phosphorylated CREB levels correlate with foetal haemoglobin in individual patients (Bailey *et al.*, 2007). Cokic *et al.* (2008) suggested a mechanistic link between NO, cGMP and HbF induction in the presence of HU since inhibition of cGMP lead to inhibition of the HU mediated HbF induction.

Although each of these models has strong evidence supporting their involvement in HbF induction, they do not explain how such a diverse group of compounds can have the same effect. Mabaera *et al.* (2008b) introduced a mechanistic model of HbF induction which integrates the activation of p38 MAPK through multiple upstream signals including DNA damage, oxidative stress (ROS), heat shock, osmotic shock, NO and inhibition of protein synthesis. The ability of most agents to activate cell stress signalling pathways that augment γ-globin gene expression and HbF production during

adult erythopoiesis, has led to the proposed cell stress signalling model of foetal haemoglobin (Mabaera *et al.*, 2008b). Based on the cell stress model, the dose of inducing agents must be high enough to activate stress signalling but not so high as to induce cell cycle arrest or apoptosis in haematopoietic precursor cells.

More recently, the integrated stress response pathway has been proposed to have an important role in erythroid cells. Under various stress conditions such as viral infection, heat shock, ROS, NO (Igarashi *et al.*, 2004) endoplasmic reticulum (ER) stress (Li and Holbrook, 2004), proteosome inhibition, inadequate nutrients and haem deficiency, the integrated stress response down-regulates general protein synthesis, through inhibition of mRNA translation following phosphorylation of eukaryotic translation initiation factor 2A (eIF2A) by kinases. Translational regulation of HRI, the only kinase to be activated by arsenite-induced oxidative stress, was recently shown to be essential in reducing excessive synthesis of globin proteins and phenotypic severities in β-thalassaemia (Han *et al.*, 2005). In the absence of HRI, the steady-state level of protein synthesis in reticulocytes is considerably increased in a haem-independent manner with the primary impact on the synthesis of α- and β-globin chains, suggesting an important role for HRI in the regulation of translational initiation in erythroid precursors (Han *et al.*, 2001). Studies on thalassaemic (*Hbb*-/-) mice have shown that activation of HRI is critical in minimizing the production and the accumulation of denatured α-globin aggregates (Han *et al.*, 2005). In addition to inhibition of protein synthesis, the second important consequence of eIF2A phosphorylation under the integrated stress response is the specific increase in translation and transcription of factors that are required for stress response. One such transcription factor is ATF4 which is up-regulated upon endoplasmic reticulum stress (Harding *et al.*, 1999), leading to the secondary transcriptional responses affecting other transcription factors such as C/EBP homologous protein-10 (Chop/GADD153) (Wang *et al.*, 1996) which in turn activate additional downstream genes such as growth arrest- and DNA damage-inducible gene (GADD) 34, leading to the induction of either corrective pathways or apoptosis (Novoa *et al.*, 2003). *Atf4-/-* mice resulted in transient foetal anaemia with relatively normal erythropoieis in adulthood while studies on *gadd34-/-* mice revealed that gadd34 was required for haemoglobin synthesis. In addition, GADD34 expression was increased upon exposure to erythropoietin and DMSO-mediated differentiation in MEL cells. Despite the lack of experimental evidence about the direct role of the integrated stress response pathway in HbF induction, it should not be discarded as a possible explanation

of the translational effects of HbF inducers due to its importance in erythroid precursor cells, its ability to activate under stress conditions the production of molecules such as NO and ROS and its ability to activate some of the transcription factors involved in p38 MAPK pathway (Mabaera *et al.*, 2008b).

1.8. The use of proteomics for the study of biological processes

Molecular medicine is currently moving beyond genomics and towards proteomics since the function of proteins is closely associated with the cellular, tissue and physiological contexts that drive biological outcomes. The rate of synthesis and half-life of proteins (Varshavsky, 1996) is regulated not only by the abundance of mRNA but also by post-transcriptional mechanisms and the regulated destruction of proteins. The mRNA levels of a particular gene do not accurately represent the amount of the respective protein expressed. It is, therefore, essential to measure protein expression directly for genome-wide analysis of biological processes (Aebersold *et al.*, 2000). In order to study biological processes at the system level, analytical tools that identify the components of the system and measure their responses to a changing environment are required. Among them proteomics, the global analysis of protein expression, is a rapidly developing technology (Bantscheff *et al.*, 2007). Quantitative proteomics have the ability to detect steady-state gene expression and perturbation-induced changes leading to a more accurate representation of protein-directed biological processes (Aebersold *et al.*, 2000).

Classical proteomic quantitative methods combine protein separation by high-resolution two-dimensional gel electrophoresis (2DE) (O'Farrell, 1975), in combination with mass spectrometry (MS) or tandem mass spectrometry (MS/MS) for identification of selected proteins. 2DE was developed before the term proteomics was even employed and entails the separation of complex protein mixtures by molecular charge in the first dimension and by mass in the second dimension. Although it provides several types of information about the proteins identified including molecular weight, PI and quantity, large-scale protein analysis studies comparing the levels of expression of proteins from yeast *S. cerevisiae*, observed that the 2DE/MS approach is fundamentally limited by its ability to detect low abundance proteins and that the number of proteins detected does not correspond to the number of genes expressed in the cell from which the sample

originated (Gygi *et al.*, 1999b). These limitations are resolved by the introduction of Liquid chromatography (LC)-MS/MS techniques.

LC-MS/MS based quantitation employs stable isotope labelling of peptides which create a specific mass tag that can be recognised by a mass spectrometer. Since a stable isotope-labelled peptide is chemically identical and behaves identically to its native counterpart during chromatographic and mass spectrometric analysis, the mass tag provides the basis for quantitation of proteins. Several methods have been developed for stable isotope labelling used in proteomics and only differ in the method of introduction of tags into proteins or peptides. The tags can be introduced metabolically (Ong *et al.*, 2002), chemically (Gygi *et al*., 1999a, Ross *et al.*, 2004) or enzymatically (Yao *et al.*, 2001) as internal standards (Ong and Mann, 2005), or by spiking known quantities of synthetic peptides into the sample as external standards (Kusmierz *et al.*, 1990). Alternatively, label-free methods are available which can provide a comparison between two or more experiments by comparing the signal intensity for any given peptide or using the number of acquired spectra matching to a peptide as an indicator for its respective amount in a given sample. Each technique has its particular strengths and weaknesses. Thus careful consideration should be placed on which method is more suitable for the type of experiment performed.

Proteomics have been used in a number of applications. Quantitative proteomics are ideally suited for the discovery of protein biomarkers in the absence of any prior knowledge of quantitative changes in protein levels, and for diagnosis of diseases such as cancers, cardiovascular diseases and diabetes among others. Comparison of the proteomic profile of healthy with disease state provides information on over or under-expressed proteins or disease-induced proteins released in the serum and insights for therapeutic interventions. However, despite the many thousand proteomic studies published so far, only a minority has provided a comprehensive quantitative description of the biological systems under investigation (Ong and Mann, 2005). It is now widely recognised that the proteome is much more complex than the genome and transcriptome (Rappsilber and Mann, 2002) and the various challenges in proteomic investigations are only beginning to be determined. Several sources of potential error exist during every step of the process that can negatively affect the results of peptide quantitation. But since quantitative proteomics is a relatively new field, no standards have been agreed on how to perform the experiments and analyse the data with statistical confidence.

Therefore further research is urgently needed to ensure quality and transparency of quantitative proteomics (Ong and Mann, 2005).

1.9. Aims and Objectives

Despite the large number of compounds identified as HbF inducers, their clinical use is limited due to low HbF inducing activity, high cytotoxicity and inability to elicit a response in all patients. Thus identification of new agents with higher efficacy and reduced toxicity is imperative. A key step in developing targeted drugs is the identification of specific molecular targets whose function can be manipulated by compounds. One approach to achieve that is the identification of molecules within signalling pathways associated with the particular function of interest, followed by rational design of molecules that alter the function of the targets to produce a desirable effect. Despite years of research, most of the key molecules and pathways that mediate the induction of HbF remain to be defined. A second approach for target identification is to screen drug libraries or test promising compounds for the ability to produce a desired cellular effect and to then determine the pathways and molecules targeted by effective agents. However, neither the molecular mechanisms nor target molecules have been definitively verified for any of the 70 HbF inducing compounds described so far. Therefore, the most direct route to identifying specific molecules for targeted drug development may involve taking advantage of these lead compounds to determine their molecular mechanism of action.

In the current project two approaches have been employed with the aim of identifying potential HbF inducers. Initially, agents that are structurally similar to compounds with known HbF inducing activity will be screened for identification of novel agents with potent HbF inducing activity. Then, the molecular pathways of a known HbF inducer will be investigated with the aim of identifying potential targets for therapeutic manipulation and target based drug design.

My immediate objectives included:

- *In vitro* screening of agents belonging to the xanthine group of substances using a mouse erythroleukaemic cell model.

- Screening of eleven derivatives of resveratrol in a human erythroleukaemic cell line with the purpose of identifying a compound which combines the HbF inducing activity of resveratrol with lower cytotoxicity.

- Screening of four potent HbF inducers: (1) Lenalidomide, an immunomodulator drug, (2) Angelicin, a compound structurally related to psoralens, (3) 5-aza-2'-deoxycytidine (decitabine), an antimetabolite nucleoside analogue and (4) Mithramycin, another DNA binding agent, in a human erythroleukaemic cell line and primary human erythroid progenitor cells with the purpose of selecting the most effective agent with regards to HbF induction.

- Investigation of the molecular mechanisms of action of the most effective agent by quantitative proteomic analysis of primary human erythroid cultures from healthy individuals and transfusion-dependent thalassaemic patients treated with the particular agent.

- Investigation of the DNA and Histone methylation patterns of the LCR and globin gene promoters in primary human erythroid cultures from healthy donors treated with the agent using Chromatin immunoprecipitation (ChIP) assay

- Investigation of changes in the mRNA levels of ten transcription factors associated with γ-globin gene expression, in primary human erythroid cultures from healthy donors and transfusion-dependent thalassaemic patients in the presence of the selected agent by quantitative PCR

- Investigation of the effect of down-regulation of 17 significantly differentially expressed proteins (identified through proteomics) in primary human erythroid cultures from healthy donors, in the absence and presence of the selected agent.

2. **MATERIALS AND METHODS**

2.1. Cell culture

2.1.1. Cell culture materials

	Description	Supplier
Media	Roswell Park Memorial institute (RPMI) 1640 Medium	GIBCO Invitrogen Inc, Paisley UK
Serum	Foetal Bovine Serum (FBS), Heat inactivated	GIBCO Invitrogen Inc, Paisley UK
Supplement	L-Glutamine 200nM (100x)	GIBCO Invitrogen Inc, Paisley UK
Supplement	Penicillin-Streptomycin (10,000 U/ml)	GIBCO Invitrogen Inc, Paisley UK
Supplement	Sodium Pyruvate	GIBCO Invitrogen Inc, Paisley UK
Antibiotic	Gentamicin (G418) 10mg/ml	GIBCO Invitrogen Inc, Paisley UK
Dye	Trypan Blue	Sigma-Aldrich, St Louis USA
Tubes	15ml Falcon tubes	Corning Inc, NY USA
Flasks	$T25cm^2$ Nuclon^{TM} Delta treated	NUNC, Roskilde Demark
Plates	6-well plates	NUNC, Roskilde Demark
Plates	12-well plate	NUNC, Roskilde Demark
Centrifuge	Eppendorf 5810 centrifuge	Eppendorf, Hamburg Germany

2.1.2. Culturing the MEL cell line

Attempts to identify pharmacological reactivators of foetal haemoglobin require screening of agents in experimental models that replicate the *in vivo* regulation of β-like globin genes. Several groups have been using cells transfected with reporter genes under the transcriptional control of the γ- and β-globin gene promoters in order to identify more specific agents (Skarpidi *et al.,* 2000, Vadolas *et al.,* 2004). The advantage of these systems is their ability to become automated and thus be used for high throughput screening of HbF inducers.

The initial screening of potential HbF inducers was performed on the mouse erythroleukaemia cell line GM979 (MEL). The GM979 cell line was modified by incorporating the $\mu LCR\beta_{pr}R_{luc}{}^{A}\gamma_{pro}F_{luc}$ construct (Figure 6) containing two luciferase proteins, Firefly and Renilla, under the control of the human γ- and β-globin promoters, respectively, as originally described by Skarpidi *et al.* (2000).

Cells were cultured in RPMI medium enriched with 10% Foetal Bovine Serum (FBS), 0.6% Glutamine, 50 U/ml of Penicillin/Streptomycin, 1% Sodium Pyruvate and 400μg/ml G418 Geneticin reagent, an aminoglycoside related to Gentamicin for selection of the clones containing the $\mu LCR\beta_{pr}R_{luc}{}^{A}\gamma_{pro}F_{luc}$ construct, in $T25cm^2$ flasks in a humidified atmosphere of 5% CO_2 at 37˚C. Cells were sub-cultured every two days, when complete media change was performed. The cells were collected in 15ml falcon

tubes and centrifuged for 5 min at 1200rpm. The supernatant was discarded and cells were re-suspended in 12ml medium at a density of $0.4x10^6$ cells/ml.

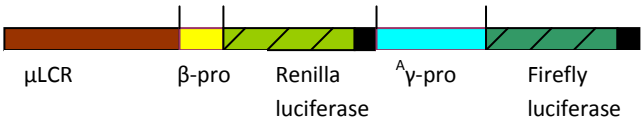


Figure 6, Dual luciferase reporter construct ($\mu LCR\beta_{pr}R_{luc}{}^{A}\gamma_{pro}F_{luc}$) transfected into the GM979 cell line. *Scarpidi et al.(2000)Blood 96(1):321-216*

2.1.3. Culturing the K562 cell line

In 1981, Rutherford *et al.* (1981) was the first to suggest the use of the human cell line K562 as a model for studying haemoglobin switching. K562 cells, originally established by Lozzio and Lozzio (1975) were derived from a patient with chronic myeloid leukaemia in terminal blast cell crisis. Studies have shown that the K562 blasts are multipotential, haematopoietic malignant cells that can spontaneously differentiate into recognisable progenitors of the erythrocytic, granulocytic and megakaryocytic series (Andersson *et al.*, 1979).

K562 cells were cultured in RPMI medium enriched with 10% Foetal Bovine Serum (FBS), 0.6% Glutamine and 50 U/ml of Penicillin/Streptomycin. Cells were maintained at 37˚C in a humified atmosphere of 5% CO_2 using Nunc T25cm^2 tissue culture flasks. Every two days, the cells were collected into 15ml falcon tubes, centrifuged at 1200rpm for 5 min and re-suspended in new media at a density of $0.3x10^6$ cells/ml.

2.2. Screening of potential agents in MEL cells

2.2.1. Treatment of MEL cells

For experimentation, the cells were collected in 15ml falcon tubes and centrifuged at 1200rpm for 5min. The supernatant was discarded and cells were washed with 1X PBS to remove any excess antibiotic. The cell numbers were estimated by Trypan Blue staining on a Haemocytometer (Hausser Scientific, Horsham, USA) and cells were seeded in 6-well plates at $5x10^4$ cells/ml in 4ml medium per well. The appropriate

concentration of each compound to be studied was added to each well. Un-treated cells were used as negative control whereas treatment with 10mM Propionic acid was used as positive control. Triplicates of each culture condition were performed and plates were incubated at 37°C in a humified atmosphere of 5% CO_2 for 4 days prior to collection of the cells for the luciferase assay.

2.2.2. Luciferase assay

2.2.2.1. Materials and Kits

	Description	Supplier
Kit	Dual-Luciferase Reporter Assay	Promega, MAD USA
Tubes	2.5ml Polystyrene tubes	BD Biosciences, Erembodegem Belgium
Luminometer	Tube luminometer	Berthold Detection System GmbH, Pforzheim Germany

2.2.2.2. Measuring luciferase activity in MEL cells

A luciferase assay was used to measure the effect of potential inducers in MEL cells. On day 4 of treatment, the cells from each well were collected into 15ml falcon tubes and centrifuged at 1200 rpm for 5 min and washed once with 1X PBS. Trypan blue staining was used to estimate cell number and viability of cells in each condition. The cells were centrifuged once more at 1200 rpm for 5min and the supernatant removed. 100µl 1x Passive Lysis buffer prepared from 5x stock solution in distilled water, was added to the re-suspended pellet and incubated at room temperature for 15 min to allow lysis of cells. The firefly luciferase reporter assay was initiated by adding 20µl of the lysed cells suspension to 100µl LARII solution (prepared by re-suspending stock LARII powder in 10ml Buffer according to manufacturer's instructions, Promega) in a 2.5 ml polystyrene tubes. The firefly luciferase activity was then measured on a tube luminometer. Addition of 100µl 1X Stop and Glow Solution (1X), prepared just before use from a 50x stock substrate, to the samples allows the quenching of firefly luciferase luminescence and concomitant activation of renilla luciferase. Renilla luminescence was quantitated with the luminometer as fast as possible.

The increase activity of the γ-globin promoter in the presence of the agent was calculated by the ratio of firefly luciferase to the total luciferase activity for each sample

where firefly intensity represented the γ-globin promoter activation and Renilla intensity, the β-globin promoter activation. In the current system, firefly luciferase activity has approximately 50% greater luminescence than renilla luciferase activity per mole and therefore multiplication of the measurement of renilla luciferase activity by a factor of 2 will adjust for the difference in the measured activity. The ratio $\gamma^F/(\gamma^F+2\beta^R)$ therefore, represents the increase in γ-globin promoter activity relative to the total increase in globin gene promoter activity.

2.3. Screening of potential agents in K562 cell line

2.3.1. Treatment of K562 cells with potential agents

Cells were counted using Trypan Blue staining and were collected in 15ml falcon tubes and centrifuged at 1200rpm (Centrifuge 5810, Eppendorf) for 5min. The supernatant was discarded and the cells were seeded in 12-well plates in 2ml per well at a density of $2x10^4$ cells/ml. The appropriate concentration of each compound to be studied was added to each well. Un-treated cells were used as a negative control and treatment with 150μM Hydroxyurea was used as a positive control. Triplicates for each culture condition studied were performed and the plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 5 days. After five days incubation with the agent, the cells were collected and cell viability was determined by Trypan Blue. The level of cellular differentiation as a result of the effect of the inducing agent was determined by Benzidine staining.

2.3.2. Benzidine staining

2.3.2.1. Materials and reagents

	Description	Supplier
Dye	Benzidine dihydrochloride	Sigma Aldrich, St Louis USA
Reagent	Glacial acetic acid	BDW VWR international Poole UK
Reagent	MilliQ Distilled water	Millipore, GaA, Darmstadt Germany
Reagent	30% Hydrogen peroxide	Merck, Darmstadt Germany

2.3.2.2. Benzidine staining procedure

Benzidine staining is the most widely used method for scoring erythroid differentiation. It is highly specific for haem-containing compounds and can thus be used for the detection of haemoglobin (Nagy *et al.*, 1995). Haemoglobin containing cells will stain blue in the presence of benzidine stain. However, benzidine staining is such a sensitive method that sometimes myeloid cells with high haem-containing enzyme-peroxidase content may stain lightly blue (Fibach, 1998).

Stock solution of the stain was prepared by the addition of 1g benzidine dihydrochloride and 14.6ml glacial acetic acid to 485.4ml of distilled water. The bottle was kept in the dark with constant stirring for a few hours until benzidine was dissolved.

A working solution was then prepared by the addition of 20µl of 30% H_2O_2 to 1ml benzidine stock solution. Equal volumes of the working solution and cells were combined and allowed to stand at room temperature for 2-3 minutes. The haemoglobin containing cells were stained blue and can be scored using a haemocytometer.

2.4. Primary human erythroid progenitor cell culture

2.4.1. Materials and reagents

	Description	Supplier
Medium	Alpha MEM 2x	Sigma-Aldrich, St Louis USA
Serum	Certified Foetal Bovine Serum US origin (1600044)	GIBCO Invitrogen Inc, Paisley UK
Supplement	Cyclosporin A 1mg/ml	Sigma-Aldrich, St Louis USA
Supplement	Bovine Serum Albumin (BSA)	Sigma-Aldrich, St Louis USA
Supplement	β-mercaptoethanol 1mM	Sigma-Aldrich, St Louis USA
Supplement	Dexamethasone 21-phosphate disodium salt	Sigma-Aldrich, St Louis USA
Supplement	Glutamine 200mM	GIBCO Invitrogen Inc, Paisley UK
Supplement	Lyophilised human Stem cell factor	GIBCO Invitrogen Inc, Paisley UK
Supplement	Human recombinant Erythropoietin	Janssen-Cilag Lt, Bucks, UK
Reagent	PBS	GIBCO Invitrogen Inc, Paisley UK
Reagent	Lympholyte M	Tebu-Bio, Le-Perray-en-Yvelines France
Tubes	50ml falcon tubes	Greiner Bio-one, Frickenhausen, Germany
Pipettes	5ml, 10ml and 25ml pipettes	Costar, Corning Inc., New York, USA
Flasks	T75cm2 flasks Nuclon^{TM} Delta treated	Nunc, Roskilde Denmark

2.4.2. Preparation of cell culture reagents

10% Bovine Serum Albumin (BSA):

25g BSA were diluted in 110ml double deionised water overnight at 4˚C in the dark using a magnetic stirrer. The following day, an equal volume (110 ml) of the double concentrated (2x) α-MEM was added to the solution. The solution was mixed for another hour at room temperature using a magnetic stirrer, filter-sterilised through a 0.22μm filter and stored at 4˚C.

Double concentrated (2x) alpha-MEM medium

1 litre powder package of α-MEM was dissolved in 0.5L of distilled water. Addition of 2.2g sodium bicarbonate changed the pH and consequently the colour of the solution into an orange-red colour. The pH was adjusted to 7.2-7.4 using sodium hydroxide solution. The medium was supplemented with 100U/100μg Penicillin/Streptomycin, filter-sterilised through a 0.22μm filter and stored at 4°C.

Conditioned Medium from H5637 cell line

Conditioned Medium was derived from the H5637 human bladder carcinoma cell line. Studies has shown that the conditioned medium contains factors including colony stimulating factor, that are capable of stimulating the proliferation and growth of human myeloid leukemic cell (Michaeli *et al.*, 1986). H5637 cells were cultured in α-MEM supplemented with Glutamine, Penicillin/Streptomycin and 10% FBS (37°C, 5% CO_2 humidified atmosphere). Once a confluent monolayer was formed following a week's incubation, the medium was collected and centrifuged at 1200rpm for 5 min. The supernatant was filter sterilised and finally stored in 15ml aliquots at -20°C.

Phase I medium

α-MEM (1x)	81%
Glutamine	1%
Penicillin/Streptomycin	5U/ml/5μg
Foetal bovine serum	10%
Cyclosporin A	1μg/ml
Conditioned medium from H5637 cell line	10%
Stem cell factor	10ng/ml

Phase II medium

α-MEM (1x)	69%
Foetal Bovine serum	30%
β-mercaptoethanol	0.01mM
Dexamethasone	0.77μM
Recombinant Erythropoietin	1U/ml
Stem cell factor	10ng/ml
BSA (10%)	1%
Penicillin/Streptomycin	5U/ml/5μg
Glutamine	2mM

2.4.3. Introduction

The two-phase method originally described by Fibach (1989) was used for culture of primary human erythroid progenitor cells from peripheral blood or buffy coat obtained from healthy individuals and thalassaemic donors.

Fibach (1998) introduced a method where cells were grown in suspension and consequently overcoming many of the limitations of semi-solid method. This culture method has the advantage of yielding large and relatively pure populations of cells and can recapitulate *in vivo* erythropoiesis. The original two-method comprises of an erythropoietin-independent phase where the erythroid committed progenitors proliferate and differentiate into more mature progenitors in the absence of erythropoietin (EPO); followed by an erythropoietin-dependent phase where the progenitors from the previous phase differentiate in the presence of erythropoietin into orthochromatic normoblasts and enucleated erythrocytes (Figure 7).

During the first phase, erythroid progenitors along with leukocytes and monocytes are isolated by density gradient centrifugation (Section 2.4.4) and grown in the presence of transcription factors such as cyclosporin A and stem cell factor (SCF) but in the absence of EPO. Cyclosporin A is responsible for the inhibition of proliferation of lymphocytes while the presence of SCF stimulates the proliferation of haematopoietic progenitors. At the end of Phase I, only the non-adherent cells in the supernatant, comprising mostly of haematopoietic progenitors, are transferred into Phase II. This ensures the elimination of non-haematopoietic cells such as monocytes and macrophages that have the tendency to attach to the culture plate. Phase II medium allows the differentiation of the erythroid progenitors into mature erythroblasts in the presence of a cocktail of factors containing dexamethasone, SCF, EPO, FBS and BSA.

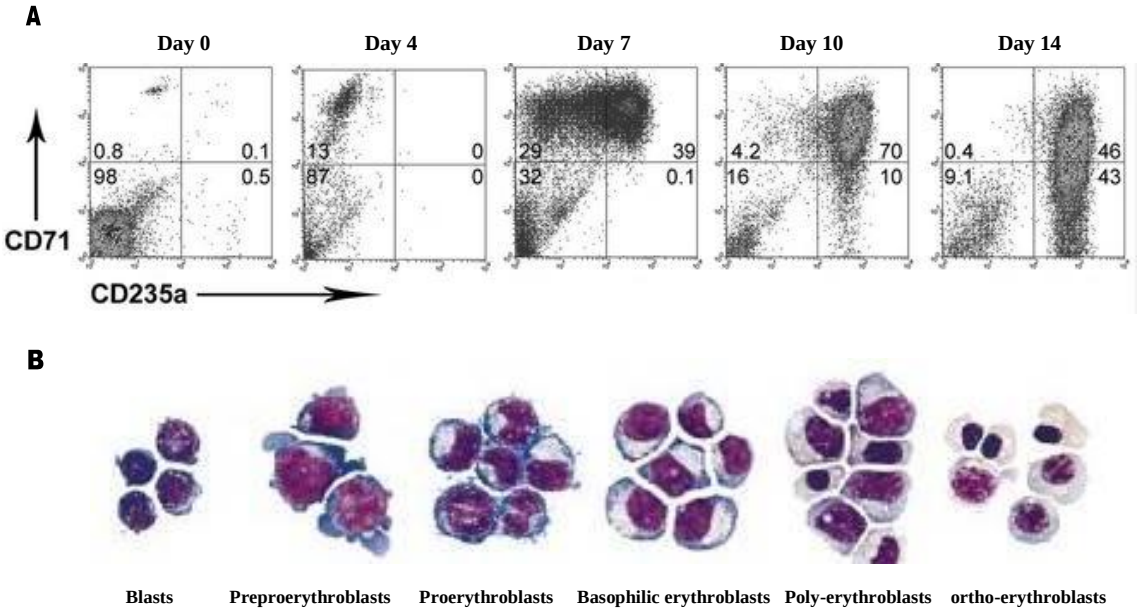


Figure 7, Maturation stages of primary human erythroid progenitor cells defined by the expression of CD71 and GPA surface antigens (A) and morphology (B). Adapted from *Lamikanra et al. (2009) PlosONE 4(12):e8446* and *Wojda et al. (2002) Blood 99(8):3005-13.*

2.4.4. Isolation of mononuclear cells from peripheral blood

Buffy coats from healthy donors were obtained from the National Blood Bank in Nicosia for the establishment of each culture. Peripheral blood (~20ml) from thalassaemic donors was provided by Dr Maria Sitarou (Thalassaemia Department, Larnaca General Hospital, Cyprus). Both the buffy coat and the peripheral blood were diluted 1:1 (v/v) with 1X PBS and gently layered on lympholyte (density of 1.0777g/ml). The lympholyte layer was added as 1/3 of the volume to a 50ml falcon tube and topped up very slowly with diluted buffy coat or blood. The tubes were then centrifuged at 2000rpm for 30 min without brake (Eppendorf centrifuge 5810, Eppendorf) at room temperature. Density gradient centrifugation separated the diluted blood into three phases, the top layer (containing platelets and plasma), the interphase layer (containing mononuclear cells) and the bottom layer (containing the red blood cells). The interphase layer containing the primary human erythroid progenitor cells with the mononuclear white blood cells was collected with a sterile 5ml pipette and transferred to a new 50ml falcon tube. The cells were then topped up to 50ml with 1X PBS and centrifuged at 1500 rpm for 8 min. The supernatant was removed and the pellet re-suspended by gentle pipetting. The cells were then washed twice with 1x PBS and centrifuged at 1200 rpm and at 1000rpm for another 8 min after the first and second

wash respectively in order to remove any platelets and red blood cells remaining in the pellet. The cells can be further washed with 1x PBS if the supernatant is still not clear. An outline of the procedure can be seen in Figure 8.

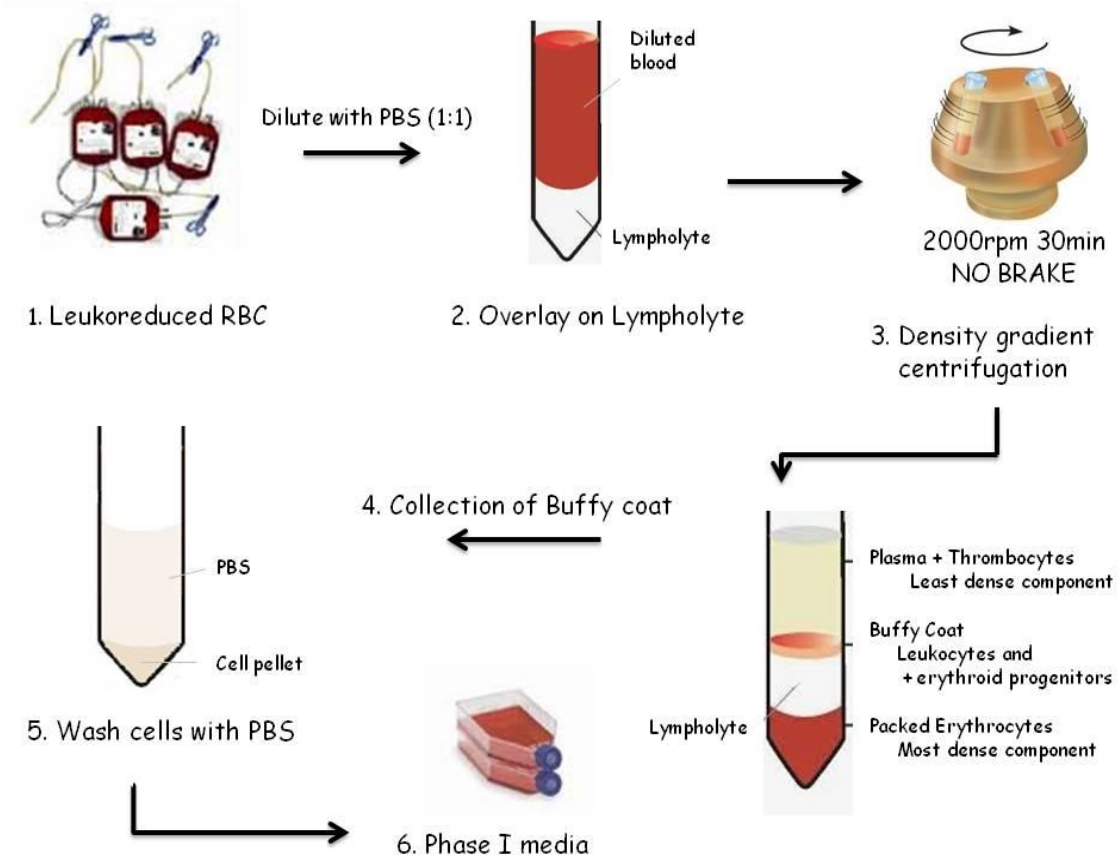


Figure 8, Outline of the process followed for isolation of mononuclear cells from peripheral blood or buffy coat

2.4.5. Culture of isolated mononuclear progenitor cells

Isolated mononuclear cells were re-suspended in Phase I medium (Day 0) and were cultured for 7 days at 37°C in 5% CO_2 humidified atmosphere. During Phase I, isolated peripheral blood mononuclear cells grow in the presence of growth factors but in the absence of EPO. On day 6 of Phase I, non-adherent cells were harvested and centrifuged at 1200rpm for 8 min. The cells were washed once with 1X PBS and the pellet was re-suspended in EPO-supplemented Phase II medium. Cells were incubated again at 37°C with 5% CO_2.

2.4.6. Treatment by potential agents

For the study of potential HbF inducers, test compounds were added to the primary human erythroid cultures on day 6-7 of Phase II. The cultured cells were re-suspended by pipetting and the number of cells in each culture flask was determined by trypan blue staining. Primary human erythroid progenitor cells were distributed into T25m^2 flasks at a concentration of ~2x10^6 cells per ml. One T25m^2 flask was set up for each concentration of the compound. The corresponding compound to be studied was then added to the corresponding flask and incubated at 37°C in 5% CO_2 for 5-6 days. Un-treated cells, cells without any agent, were used as negative controls, and cells treated with 150μM hydroxyurea or 10mM propionic acid were used as positive controls.

On day 12 of Phase II (following 5-6 days incubation with the agent), the cultures were disrupted by pipetting and cell numbers determined by trypan blue staining. Cells were then collected in 15ml falcons and washed twice with 1x PBS. Cell pellets were either snap-frozen in liquid nitrogen for analysis by cation-exchange HPLC for haemoglobin levels and Western blot analysis, or re-suspended in 1ml TriFast reagent and stored at -80°C for RNA extraction and analysis by real-time PCR for globin gene expression.

2.5. Cytospin

2.5.1. Materials and reagents

	Description	Supplier
Slides	Menzel-Gläser SuperFrost slides	Thermo Fisher Scientific, MA USA
Centrifuge	Cellspin II cytocentrifuge	Tharmac, Waldsolms, Germany
Materials	Filter paper	Tharmac, Waldsolms, Germany
Dye	Hema 'Gurr' rapid staining set for Haematology	BHD, VWR international, Poole, UK
Fixative	DPX mountant for histology	Sigma-Aldrich, St Louis USA
Slides	Microscope cover glass	Deckglaser, Germany

2.5.2. Cytospin preparation

During Phase II, the progress of the culture and the stage of differentiation of the cells can be assessed by cytospin preparation. Cells were seeded onto Menzel-Gläser SuperFrost slides by the use of Cellspin II cytocentrifuge. The slide, filter paper and funnel were inserted into the clip in that order and sealed in the correct position. Depending on the density of the cultures, 150-300μl of each culture was placed into the

funnel opening and the whole clip was placed in the cyto-centrifuge at an angle of 45°. Once the clips were arranged in balance within the chamber, the cells were centrifuged at 1000rpm for 3 minutes. The funnel and the filter paper were then removed from the slide without disrupting the cell smear and the slide was air-dried.

2.5.3. Staining of cytopsins

The dried slides were treated with Hema 'Gurr' rapid staining set for Haematology where cells were fixed using a fixative solution containing CH_3OH, followed by staining with Gurr staining reagent 1 (Eosin Y) and staining with Gurr staining reagent 2 (Methylene blue + Azure II). The purple colour obtained by cell nuclei after staining is due to molecular interaction between eosin Y and the methylene blue/azure II-DNA complex. After drying, the cytospin were mounted with a cover slide.

2.6. Flow Cytometry

2.6.1. Antibodies and instrumentation

	Description	Supplier
Flow cytometer	CyFlow Cube 8	Partec, Görlitz, Germany
Antibody	PE anti-human CD235a (HIR2 clone)	BioLegend, CA USA
Antibody	APC anti-human CD71 (CY1G4 clone)	BioLegend, CA USA

2.6.2. Introduction

The stage of differentiation of the primary human erythroid cells can be determined by immunostaining with fluorescently labelled monoclonal antibodies directed against the surface antigen Glycophorin A (CD235a) and transferrin receptor (CD71) (Figure 7). Anti-CD235a-PE (Phycoerythrin) is an antibody raised against Glycophorin A, a major sialoglycoprotein expressed on red cell membrane and erythroid precursors, and is useful in determining erythroid cell development. Anti-CD71-APC (Allophycocyanine) is an antibody raised against transferrin receptor, a homodimeric transmembrane glycoprotein that is expressed on proliferating cells. Analysis of cell surface antigen expression of differentiating erythroid progenitors and fluorescence-activated cell sorting of primary human samples have demonstrated that early erythroid progenitors such as BFU-E and CFU-E cells express CD71 marker but lack GPA expression. The

expression of GPA progressively increases as immature erythroblasts differentiate while maintaining their CD71 expression. Terminally differentiated erythroblasts show up-regulated GPA expression and severely decreased CD71 expression (van den Akker *et al.*, 2010, Li *et al.*, 2014). The lack of expression of GPA in BFU-E or CFU-E cells and high expression in mature erythroblasts confirms its function as a differentiation marker.

2.6.3. Phenotyping of Primary human erythroid cultures

Approximately 1-2x10^6 cells were labelled with 1.5µl anti-CD71 and anti-GPA monoclonal antibodies in 100µl PBS for 30 minutes in the dark. The cells were then washed twice with PBS at 1000rpm for 5 min, re-suspended in 1ml PBS and analysed based on their surface antigen positivity using CyFlow Cube 8 flow cytometry. Cells were excited at 488nm and detected in FL2 (PE) and in FL3 (APC) in logarithmic scale.

2.7. Cation-exchange High Performance Liquid Chromatography (HPLC)

2.7.1. Materials

	Description	Supplier
Reagent	LiChrosolv Water	Merck KGaA, Darmstadt Germany
Reagent	Bi-Tris	Sigma Aldrich Co, St Louis USA
Reagent	Potassium Cyanide	Sigma Aldrich Co, St Louis USA
Reagent	EMSURE NaCl	Merck KGaA, Darmstadt Germany
Centrifuge	Eppendorf 5415R	Eppendorf, Hamburg, Germany
Column	20x4.6mm PolyCAT A™	PolyLC, Columbia USA
Instrument	Shimadzu LCsolution instrument	Shimadzu Corporation, Kyoto, Japan
Reagent	Lymphocheck™ haemoglobin A_2 control	BioRad laboratories Inc, CA USA

HPLC buffers:

	Bis-Tris	KCN	NaCl	pH
Phase A	10mM	1mM	-	6.87
Phase B	10mM	1mM	200mM	6.57

2.7.2. Introduction

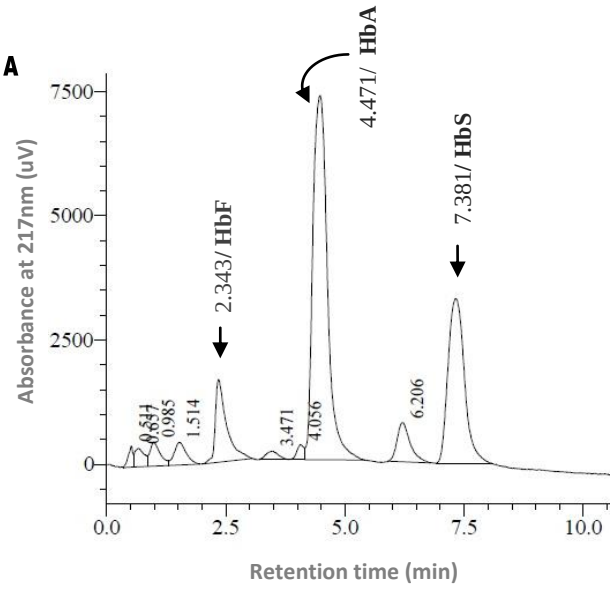
Cation-exchange chromatography has been previously used for separation of minor haemoglobin types including modified HbA components and was showed to be a good method for separation of HbF from the remaining HbA variants (Gupta and Hanash, 1983, Ou and Rognerud, 1993). In cation exchange chromatography, positively charged molecules are attracted to a negatively charged solid support through ionic interactions and are released with increasing concentration of salt solution. Molecules with less charge and thus weaker interactions are eluted first.

2.7.3. Procedure

The percentage of HbF was determined by cation exchange High Performance Liquid Chromatography (HPLC) coupled with a 3.5x0.46cm HPLC column packed with 5µm silica coated porous particles, as described previously by Ou and Rognerud (1993).

Around 3-5x10^6 primary human erythroid progenitor cells were collected on Day 12 of Phase II, washed once with PBS and the cell pellet was snap-frozen in liquid nitrogen and stored at -80°C. On the day of experimentation, the cell pellets were lysed in ~15µl HPLC water per 1x10^6 cells by vortexing and incubated for 20 min on ice. The cell debri was pelleted by brief centrifugation and the supernatant was topped up with equal volumes of mobile phase A buffer. Samples were then ready for analysis by cation exchange HPLC analysis. The different haemoglobin species from the cell lysates were separated on a 20 x 4.6mm PolyCAT A™ HPLC column fitted onto the Shimadzu LCsolution instrument. Elution of haemoglobin was performed by increasing the mobile phase B from 12% to 40% and 100% in mobile phase A at 8 and 12 min respectively, and reduced to 12% again at 13min. The column was re-equilibrated with 12% mobile phase B for at least 7 min before the application of the next sample. Haemoglobin proteins were detected at 217nm. Lyophilised human whole blood based control (Lymphocheck™ haemoglobin A_2 control) was used as a reference for the retention time of each haemoglobin type (Figure 9). The Lyophilised control contains defined percentages of HbA_2, HbF and HbS.

A



B

Haemoglobin	*Retention Time*	*Percentage*
HbF	2.343	8.8
HbA	4.471	50.8
HbF	7,381	25.9

Figure 9, Analysis of the lyophilised human whole blood control by Cation exchange HPLC. The chromatograph showed the retention time of peaks corresponding to each haemoglobin (A) and the percentage of each haemoglobin (B) as calculated by the area under each peak

2.8. RNA purification

2.8.1. Materials

	Description	Supplier
Kit	RNeasy Mini kit	Qiagen GmbH, Hilden, Germany
Material	0.8 mm x 40mm 21G x1.5" 2ml syringe	Becton Dickinson, NJ USA
Reagent	EMSURE Ethanol	Merck KGaA, Darmstadt Germany
Reagent	peqGOLD Trifast	Peqlab, Sarisbury Green, UK
Reagent	EMSURE Chloroform	Merck KGaA, Darmstadt Germany
Reagent	Isopropanol	Sigma Aldrich Co, St Louis USA
Centrifuge	Eppendorf 5415R	Eppendorf, Hamburg, Germany
Instrument	NanoDrop 1000 Spectrophotometer	Thermo Fisher Scientific, MA USA
Reagent	Agarose	Sigma Aldrich Co, St Louis USA
Reagent	Gel loading Dye (6x)	New England BiolAbs, MA USA
Reagent	100pb DNA ladder	New England BioLabs, MA USA
Dye	Ethidium Bromide	BHD VWR international, Poole, UK

2.8.2. RNA purification using the Qiagen RNeasy Mini Kit

On day 12 of Phase II, primary human erythroid progenitor cells from each experimental condition were collected and centrifuged at 1200rpm for 5 min and the cells washed once with 1xPBS. RNA was extracted and purified using the RNeasy Mini kit according to the manufacturer's instructions. Briefly, 600µl of guanidine isothiocynate-containing RLT Buffer (provided by the kit) was added to the cell pellet and the solution was mixed by pipetting. The solution was then homogenised by passing the lysate at least 5 times through a blunt 20-gauge needle fitted on an RNase-free

syringe. An equal volume of 70% ethanol was then added to the homogenised lysate and mixed well by pipetting. The solution was then transferred to an RNeasy spin column which was placed into a 2ml collection tube. The tube was then centrifuged at 10000 rpm for 15 sec. The flow through was discarded and 700µl of Buffer RW1, a guanidine salt-containing buffer responsible for removal of proteins and fatty acids, was added to the RNeasy spin column. The column was incubated for 5 min at room temperature and centrifuged at 10000 rpm for 15 sec. Once the flow through was discarded, each RNeasy spin column was washed twice with 500µl Buffer RPE and centrifuged at 10000rpm for 15 sec to wash the spin column membrane effectively. Finally the RNeasy spin column was placed in a new 1.5ml collection tube and 50µL of RNase-free water was added. The columns were centrifuged at 10000rpm for 1 min and the RNA eluent stored at -20˚C.

2.8.3. Manual RNA purification with Trifast reagent

If the RNA extraction was not done on the same day, the cell pellets were re-suspended in peqGOLD Trifast reagent, a guanidine isothiocyanate and phenol containing reagent, and stored at -80°C. On the day of extraction, the cell pellets were defrosted and allowed to stand for 3 min at room temperature. 200µl of chloroform was added to each tube and the samples were shaken vigorously for 10-15 seconds. The samples were then incubated at room temperature for 3 min and centrifuged for 15 min at 1200rpm at 4°C. After centrifugation, the aqueous layer was collected into a new 1.5ml eppendorf and RNA precipitated with 500µl isopropanol. The samples were mixed by inversion and incubated at room temperature for 10 minutes. The samples were then centrifuged for 10 minutes at 1200 rpm at 4°C. Isopropanol was removed and the RNA pellet washed with 75% ethanol overnight at -80°C. On the following day, the samples were centrifuged for 20min at 1200 rpm at 4°C to remove any ethanol present, the pellets were air-dried and re-suspended in 10µl DEPC-treated water.

2.8.4. Spectrophotometric RNA quantitation

The RNA concentration was determined by using the NanoDrop 1000 Spectrophotometer. 1µl of the extracted RNA was loaded onto the NanoDrop and the concentration of the RNA was calculated automatically by the machine based on

absorbance calculated at 260nm and 280nm utilizing a 0.2mm path length. The purity of the RNA was considered acceptable when the 260/280 ratio was ~2.0 and the 260/230 ratio was within the 1.8-2.2 range.

2.8.5. Gel electrophoresis

To check the integrity of the extracted RNA, 1µl of RNA, mixed with 8µl H_2O and 1µl loading dye, was loaded onto 1% agarose gel and run at 95V for 50 min. Once electrophoresis was done, the gel was stained with Ethidium Bromide. Under exposure with UV light, an image of the gel was taken with the Vilber Lourmat Gel documentation system (Figure 10).

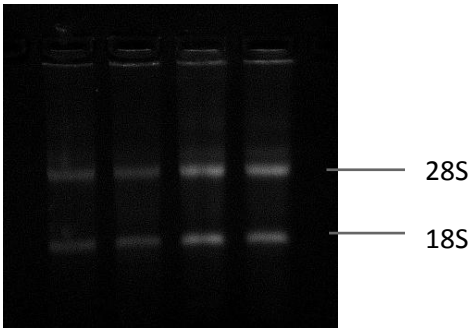


Figure 10, Verification of RNA quality by gel electrophoresis. Good quality RNA will show two bands when loaded onto 1% agarose gel, corresponding to the 28S and 18S ribosomal RNAs.

2.9. Reverse transcription Polymerase Chain Reaction (RT-PCR)

2.9.1. Materials

	Description	Supplier
Kit	TaqMan Reverse Transcription Reagents	Applied Biosystems, New Jersey USA
Reagent	RNase free water	Sigma-Aldrich, St Louis USA
PCR machine	Veriti Thermal Cycler	Applied Biosystems, New Jersey USA

2.9.2. cDNA synthesis

Synthesis of cDNA from total RNA samples is the first step in the two-step process of quantitation of gene expression levels in primary human erythroid progenitor cells.

Complementary DNA (cDNA) was synthesised from 1µg RNA using the TaqMan Reverse Transcription Reagents kit with random hexamers from Applied Biosystems. 1µg RNA was added to 1x reaction mixture (Table 1) according to manufacturer's instructions. The reaction mixture was made up to a total volume of 25µL with RNase-free water. Reverse transcriptase PCR was performed on Thermal Cycler Veritin with thermal cycling of 10 min at 25˚C, 30 min at 48˚C, 5 min at 95˚C and finally cooled to 4˚C.

Quantitation of cDNA was performed using the NanoDrop 1000 Spectrophotometer as before (Section 2.8.4). The concentration of cDNA was calculated based on the absorbance calculated at 260nm and 280nm utilizing the 0.2mm path length.

Table 1, Reverse transcription reaction mixture (1x)

Reagent	**Final concentration**	**Volume (µl)/reaction**
10X TaqMan RT buffer	1X	2.5
25mM $MgCl_2$	5.5mM	5.5
Deoxyribonucleotides (dNTPs) (2.5mM)	500µM	5
Random Hexamers (50µM)	2.5µM	1.25
RNase inhibitors (20U/µL)	0.4U/µl	0.5
MultiScribe™ Reverse Transcriptase (50U/µL)	1.25U/µl	0.625

2.10. Quantitative Real-time PCR

2.10.1. Multiplex Real-time PCR

Goblin gene expression levels in primary human erythroid progenitor cells were investigated using a multiplex real-time-PCR assay. Quantitative real-time PCR (qRT-PCR) assay was performed on 50ng cDNA using the CFX96 Touch Real-time Detection System (BioRad laboratories Inc, CA USA) coupled with iQ™ Multiplex Powermix (BioRad laboratories Inc, CA USA). The powermix contains an antibody mediated automatic hot start iTaq DNA polymerase. Upon heat activation at 95°C, the

antibodies which are keeping the polymerase inactive become irreversibly denatured, releasing in this way the fully active and unmodified iTaq DNA polymerase. This enables the specific and efficient primer extension with the convenience of room temperature reaction assembly.

The multiplex reaction, through hydrolysis of each dual fluorescently labelled, target-specific oligonucleotide probes, allows the detection of the α-, β- and γ-globin genes and the endogenous Glyceraldehyde-3-phosphotate dehydrogenase (GAPDH) gene in the same reaction. On the intact probe, emission of the reporter dyes (HEX, FAM, Texas red and Quasar) at the 5' end is quenched by resonance energy transfer to the quencher dye (BHQ1-3) at the 3' end. During hydrolysis, the reporter is released and separated from the quencher causing increase in the fluorescence. All the primers and probes (Table 2) were synthesised by Metabion GmbH (Martinsried, Germany) apart from the GAPDH probe which was synthesised by Biosearch Technologies (Biosearch Technologies, CA, USA). The gene expression data were normalised to individual GAPDH expression for relative quantification.

50ng cDNA was used as a template for each reaction with 1x iQ multiplex powermix. α-globin primers were used at a final concentration of 0.075μM, β- and γ-globin gene primers at a final concentration of 0.3μM, GAPDH primers at a final concentration of 0.79μM and all probes at a final concentration of 0.2μM, in a total reaction volume of 10μl (Table 3). Samples were loaded onto a 96-well plate with triplicates for each reaction.

Amplification was performed using the following cycle conditions: initial denaturation at 95°C for 30sec followed by 50 cycles of 10s at 95˚C and 30sec at 60˚C. Relative quantification was performed according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The relative expression of the target genes in the treated samples was expressed relative to the expression of the same genes in the un-treated samples as $2^{-\Delta\Delta CT}$.

2.10.2. Quantitative Real-time PCR coupled with SYBR green

The gene expression levels of 10 erythroid related genes were investigated by singleplex Real-time PCR on the ABI 7900HT Fast Real-time PCR System with the Fast 96-well block module (Applied Biosystems, New Jersey, USA) coupled with SYBR green

technology (Applied Biosystems, New Jersey, USA). All primers were purchased from Metabion (Metabion GmbH, Martinsried, Germany) (Table 4).

50ng of cDNA was used as template in a 25µl total volume reaction mixture (Table 5) with 1X SYBR Green PCR master mix. The SYBR green master mix consists of SYBR green I Dye, AmpliTaq Gold DNA polymerase, dNTPs, dUTP and ROX as the passive reference. Direct detection of PCR product formation is monitored by measuring the increase in fluorescence caused by the incorporation of SYBR green dye to the newly formed double stranded DNA in real-time.

Amplification and real-time data acquisition was performed using the following cycle conditions: initial denaturation at 95˚C for 10 min, followed by 40 cycles of 15s at 95˚C and 1 min at 60˚C. Target quantities were normalised to the ribosomal protein GAPDH. Relative quantification was performed according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The relative expression of target gene in the treated samples is expressed relative to the expression of the same gene in un-treated samples, rendering the un-treated sample as a ratio of 1.

Table 2, Primer and probe sequences for α, β and γ-globin genes used in the multiplex qRT-PCR

	Sequence (5'-3')	**PCR product size (bp)**
γ-globin primer F	TGACAAGCTGCATGTGGATC	85
γ-globin primer R	TTCTTTGCCGAAATGGATTGC	
γ-globin probe	FAM-CACCAGCACATTTCCCAGGAGC-BHQ-1	
β-globin primer F	GGGCACCTTTGCCACAC	124
β-globin primer R	GGTGAATTCTTTGCCAAAGTGAT	
β-globin probe	TEXAS RED-ACGTTGCCCAGGAGCCTGAAG-BHQ-2	
α-globin primer F	CGACAAGACCAACGTCAAGG	109
α-globin primer R	GGTCTTGGTGGTGGGGAAG	
α-globin probe	HEX-ACATCCTCTCCAGGGCCYCCG-BHQ-1	
GAPDH F	TGCACCACCAACTGCTTAGC	87
GAPDH R	GGCATGGACTGTGGTCATGAG	
GAPDH probe	QUASAR 705-CCCCTGGCCAAGGTCATCCATG-BHQ-3	

Table 3, Multiplex real-time PCR reaction mix

Reagent	Volume per reaction	Final concentration
iQ multiplex powermix (2x)	5µl	1x
α-globin primer pair (15µM)	0.05µl	0.075µM
β-globin primer pair (15µM)	0.2µl	0.3µM
γ-globin primer pair (15µM)	0.2µl	0.3µM
GAPDH primer pair (15µM)	0.53µl	0.8µM
α-globin probe (10µM)	0.2µl	0.2µM
β-globin probe (10µM)	0.2µl	0.2µM
γ-globin probe (10µM)	0.2µl	0.2µM
GAPDH probe (10µM)	0.2µl	0.2µM
H_2O	0.24µl	

Table 4, Primer sequences used in the qRT- PCR for quantitation of the gene expression levels before and after treatment of primary human erythroid progenitor cells with 300nM decitabine. The primers were designed to span exon-exon junctions using the Refseq mRNA database in NCBI Primer BLAST.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
BCL11a	TGCCCCGCAGGGTATTTGTAA	CCTGAAGGGATACCAACCCG
HMOX1	TGCTGACCCATGACACCAAG	GGGCAGAATCTTGCACTTTGTT
SP1	CTATAGCAAATGCCCCAGGT	TCTGGGCTGTTTTCTCCTTC
KLF1	CCCTCCATCAGCACACTGAC	GCCACCACTTGAGGAAGTCAT
SOX6	AGTTCTTTACTGTGGGGCAAC	CCGCCATCTGTCTTCATAC
MYB	CGCAGCCATTCAGAGACACTA	AGCTGCATGTGTGGTTCTGT
NFR2	GTCCCAGCAGGACATGGATTT	AGCTCATACTCTTTCCGTCGC
MBD2	TCCAAGTGGTAAGAAGTTCAGAAG	CAAGTCTGGTTTACCCTTATTTTGA
GATA1	GACACTCCCCAGTCTTTCAGG	CTCAGCCGCTCTGTCTTCAA
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

Table 5, Singleplex Real-time PCR reaction mixture

Reagent	Final concentration	Volume (µl)/reaction
SYBR green mastermix (2x)	1x	12.5
Primers (5µM)	0.3µM	1.5
RNase, DNase-free Water	-	8.5µl

2.11. Western blotting

2.11.1. Materials and solution composition

Cytoplasmic lysis buffer (Buffer A)

Material	Final concentration	Supplier
Hepes-KOH ph 7.9	10mM	Scharlau S.L., Mas d'En Cisa Spain
$MgCl_2$	1.5mM	Sigma-Aldrich, St Louis USA
KCL	10mM	Scharlau S.L., Mas d'En Cisa Spain

RIPA nuclear extraction buffer

Material	Final concentration	Supplier
Tris-HCL pH 7.4	20mM	Sigma Aldrich, St Louis USA
NaCl	150mM	Sigma Aldrich, St Louis USA
NP40	1%	Thermo scientific,
SDS (20%)	0.1%	Fisher Scientific, St Louis USA
Sodium Deoxycholate	0.5%	Sigma Aldrich Co, St Louis USA
EDTA	5mM	USB Corporation, OH USA

Bradford protein assay

Material	Description	Supplier
Reagent	Commasie Plus Bradford reagent	Thermo Fisher Scientific, MA USA
Cuvettes	1.5ml semi-micro photometric	Brand Gmbh & Co, Wertheim Germany
Spectrophotometer	Evolution 201/220 UV-Visible	Thermo Fisher Scientific, MA USA

Resolving gel recipe and reagents:

Material	Volumes for 10% gel	Volumes for 12% gel	Supplier
40% acrylamide	1.25ml	1.5ml	BioRad laboratories Inc, CA USA
1.5M Tris (pH8.8)	1.25ml	1.25ml	Sigma Aldrich, St Louis USA
MilliQ water	2.4ml	2.15ml	EMD Millipore Darmstadt, Germany
20% SDS	25µl	25µl	Fisher Scientific, St Louis USA
30% APS	16.7µL	16.75µl	Merck KGaA, Darmstadt, Germany
TEMED	5µl	5µl	Merck KGaA, Darmstadt, Germany

Stacking gel recipe and reagents (per two gels):

Material	Volumes	Supplier
MilliQ water	3.0743ml	EMD Millipore Darmstadt, Germany
40% acrylamide	0.625ml	BioRad laboratories Inc, CA USA
0.5M Tris (pH6.8)	1.26ml	Sigma Aldrich, St Louis USA
20% SDS	25µl	Fisher Scientific, St Louis USA
30% APS	16.6µl	Merck KGaA, Darmstadt, Germany
TEMED	5µl	Merck KGaA, Darmstadt, Germany

SDS-PAGE materials:

	Description	Supplier
Reagent	BlueStar Prestained Protein marker	Nippon Genetics Europe GmbH, Düren Germany
Material	0.45μm nitrocellulose membrane	BioRad laboratories Inc, CA USA
Stain	Ponceau stain	Sigma Aldrich, St Louis USA
Reagent	Bovine serum albumin	Sigma Aldrich, St Louis USA
Reagent	ECL Western Blotting Detection reagent	GE Healthcare, Buckinghamshire UK
Instrument	UVP BioSpectrum Imaging System	UVP, Cambridge UK

1x Running Buffer

Material	Amount per 1L	Supplier
Tris	3g/L	Sigma Aldrich, St Louis USA
Glycine	14.42g/L	Fisher Scientific, Leicester UK
SDS (20%)	5ml	Fisher Scientific, St Louis USA

1x Transfer buffer

Material	Amount per 1L
Tris	1.51g
Glycine	7.21g

2x Sample Buffer

Material	Final concentration	Supplier
Tris-HCL pH6.8	0.125M	Sigma Aldrich, St Louis USA
SDS (20%)	2%	Fisher Scientific, St Louis USA
Glycerol	21%	Merck Chemicals Ltd, Nottingham UK
B-mercaptoethanol	4%	Sigma-Aldrich, St Louis USA
Bromophenol Blue	0.005%	Sigma-Aldrich, St Louis USA

1x Tris-buffered saline (TBS) – 0.05% Tween 20

Material	Final concentration	Supplier
10x TBS	10%	-
Tween 20 (10%)	0.5%	Sigma-Aldrich, St Louis USA

10x TBS

Material	Amount per 1L	Supplier
Tris	12.1g	Sigma Aldrich, St Louis USA
NaCl	87.7g	Sigma Aldrich, St Louis USA
HCL (37%)	7ml	Sigma Aldrich, St Louis USA

Table 6, Antibodies used in Western blot analysis

Antibody	Type	Host	Detected protein MW (kDa)	Dilution	Supplier	Catalogue No
Haemoglobin γ	1^o	Mouse	18	1:1000	Santa-cruz Biotechnology Inc, Heidelberg Germany	Sc-21756
GAPDH (6C5)	1^o	Mouse	37	1:1000	Santa-cruz Biotechnology Inc, Heidelberg Germany	Sc-32233
Goat Anti-mouse IgG HRP	2^o	Goat	-	1:10000	Jackson ImmunoResearch, PA USA	115-035-003

2.11.2. Nuclear and Cytoplasmic protein extraction

Primary human erythroid cultures were collected at day 12 of Phase II and washed twice with PBS. Every $1x10^6$ primary human erythroid progenitor cells were re-suspended in 20µl of Buffer A (Table 6) for preparation of cytoplasmic extracts. The samples were incubated on ice for 20min, vortexed and centrifuged at 12000rpm for 10min. Equal volumes of sample buffer were added to the supernatant (cytoplasmic extract) and stored at -20°C if not used on the same day. The residual cell pellet which comprises mostly of cell nuclei was then re-suspended in 8µl of RIPA buffer for every $1x10^6$ cells. Samples were incubated on ice once more for 20min, vortexed and centrifuged at 12000rpm for another 10 min. An equal volume of samples buffer was added to the supernatant (nuclear extract) and stored at -20°C. Cell pellets could also be stored at -80°C before extraction.

2.11.3. Protein quantitation by Bradford assay

The protein concentration of each of the cell extracts was determined using the Coomassie (Bradford) Protein assay kit, a colourimetric method for total protein quantitation. Binding of proteins on Comassie reagent causes a shift in absorbance from red to blue at OD_{595} allowing the determination of the protein concentration for each sample.

10µl of 10-fold dilutions of each sample were combined with 990µl Commasie Plus Bradford reagent in 1.5ml semi-micro photometric cuvettes and incubated briefly at room temperature. Absorbance was measured at 595nm wavelength using the Evolution 201/220 UV-Visible Spectrophotometer. Protein concentrations were estimated by

reference to an absorbance standard curve obtained using a series of BSA standard protein dilutions (0, 0.2, 0.4, 0.6, 0.8, 1mg/ml) which were assayed alongside the unkown samples. Due to the non-linear colour response of coomassie with increasing protein concentrations, a standard curve was constructed for every assay carried out to ensure accurate protein determination.

2.11.4. Sodium dodecyl sulphate – Polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins in the nuclear and cytoplasmic extracts were separated by Sodium dodecyl sulphate (SDS) - polyacrylamide gel electrophoresis (PAGE). SDS is responsible for the denaturation of the proteins which can then travel through the polyacrylamide polymer depending only on their size in the presence of electricity. The proteins were separated using a discontinuous gel system comprising of 5% stacking gel and 10-12% resolving polyacrylamide gel. Acrylamide gels were prepared from a stock solution of 40% acrylamide and bis-acrylamide solution in a 37.5:1 ratio. Acrylamide monomers polymerise in the presence of 0.01% (by volume) tetramethyl-ethylenediamine (TEMED) and 0.01% ammonium persulphate (APS). Casting frames were set up by clamping two glass plates together separated by a spacer and placed on the casting stand. The resolving gel was prepared as described in section 2.11.1 and was used to fill the space between the two glass plates on the casting frames. The gel was allowed to polymerise for 15-30min. The stacking gel was prepared as described in section 2.11.1. Once the resolving gel was set, the stacking gel was poured on top and the comb used to form the wells was immediately inserted between the glass plates.

10-15μg of nuclear and 5-10μg cytoplasmic extracts were diluted with equal volumes of 2x sample buffer and boiled for 10min at 95°C before being loaded onto the SDS-PAGE gel along with 4μl of BlueStar Prestained Protein marker. The gels were then run at a constant current of 45mA until the bromophenol blue in the sample buffer reached the bottom of the gel. Proteins were then transferred onto a 0.45μm nitrocellulose membrane using the Criterion Blotter Tank transfer. The transfer sandwich containing the gel and the nitrocellulose membrane was set up submerged into the transfer buffer to allow equilibration of the membrane and gel and to reduce the formation of air bubbles. Pieces of filter paper and nitrocellulose membrane, cut to the size of the gel, were pre-soaked in transfer buffer and added to the transfer cassette starting from the

black/cathode in the following order: pad, filter paper, gel, membrane, filter paper and pad. Protein transfer was performed at 300mA for 2 hours on ice. Following the transfer, the membrane was stained with Ponceau stain for quick quantitation of the protein load of each sample and then washed with dH_2O and 1x TBS/0.05% Tween 20. The membrane was then blocked for 1 hour in 1% BSA in 1xTBS/0.05% Tween 20 and incubated in primary antibody overnight at 4°C. On the following day, the membrane was washed 3 times for 10min in TBS/0.05% Tween20 on a shaker and was incubated with the secondary antibody in TBS/0.05% Tween 20 with 5% (w/v) non-fat dry milk for 1 hour at room temperature. The membrane was washed 3 times for 10min in TBS/0.05% Tween 20 and developed using the ECL Western Blotting Detection reagent. Equal volumes of the ECL buffer A and buffer B were mixed and allowed to reach room temperature before being applied on the membrane for 3 min. Once the membrane was developed, quantitation was performed using the UVP BioSpectrum Imaging System.

2.12. Chromatin Immunoprecipitation (ChIP)

2.12.1. Materials

Materials	Description	Supplier
Reagent	Formaldehyde 37% (w/w)	Scharlau S.L., Mas d'En Cisa Spain
Kit	Shearing ChIP kit	Diagenode s.a., Ougree, Belgium
Reagent	Complete Protease inhibitor cocktail	Diagnostics, Basel Switzerland
Enzyme	Proteinase	Diagenode s.a., Ougree, Belgium
Beads	Protein A Agarose/Salmon sperm DNA beads	Millipore, GaA, Darmstadt Germany
Reagent	Bovine serum albumin	Sigma-Aldrich, St Louis USA
Reagent	Phenol/chloroform	Sigma-Aldrich, St Louis USA
Reagent	Glycogen (20μg/ml)	Invitrogen Inc, Paisley UK
Reagent	Isopropanol	Sigma-Aldrich, St Louis USA
Reagent	EMSURE Ethanol	Merck KGaA, Darmstadt Germany
Instrument	Bioruptor Pico sonicator	Diagenode s.a., Ougree, Belgium

Low salt wash buffer:

Material	Final concentration
SDS (20%)	0.1%
Triton X-100	1%
EDTA	2mM
Tris-HCL pH8.1	20mM
NaCl	150mM

High salt wash buffer:

Material	Final concentration
SDS (20%)	0.1%
Triton X-100	1%
EDTA	2mM
Tris-HCL pH8.1	20mM
NaCl	200mM

Tris-EDTA (TE) buffer:

Material	Final Concentration
Tris-HCL ph*.1	10mM
EDTA	1mM

Lithium Cloride (LiCl) wash buffer:

Material	Final concentration
LiCl	0.25M
NP-40	1%
DOC	1%
EDTA	1mM
Tris-HCLpH8.1	20mM

Elution Buffer:

Material	Final concentration
SDS (20%)	1%
$NaHCO_3$	0.1M

2.12.2. Introduction

Decitabine, a DNA methyltransferase inhibitor, was found to hypomethylate CpG nucleotides in the foetal globin genes in baboons (Saunthararajah *et al.*, 2004) and was initially suggested that γ-globin induction was due to DNA hypomethylation by the agent. We therefore investigated DNA and histone methylation in primary human

erythroid cultures from healthy donors in the presence and absence of decitabine, using antibodies against Methyl-CpG-binding protein 2 (MeCP2) and Histone 3 Lysine 4 dimethylation (H3K4me2), respectively. Dimethylation of Histone 3 at Lysine 4 represents a marker of active chromatin along with H3 Lysine 36 methylation and H3 acetylation (Kiefer *et al.*, 2008) and usually peaks early in active genes (Kim *et al.*, 2007). MeCP2 binds to a single symmetrically methylated CpG pair and methylated DNA and is thus capable of inhibiting transcription in gene promoters. It is a single polypeptide that contains a MBD and a transcriptional repression domain (TRD). MeCp2 binds to methylated DNA via its MBD and recruits the Sin3-histone deacetylase complex via its TRD, leading to deacetylation of methylated chromatin and subsequent transcriptional repression (Hendrich and Bird, 1998).

2.12.3. Chromatin shearing

Approximately $20x10^6$ primary human erythroid progenitor cells were cross-linked using 1% formaldehyde for 10 minutes at room temperature. Cross-linking was stopped by the addition of 1.25M Glycine and incubating for 5 min at room temperature. The cross-linked cells were centrifuged at 1500rpm for 5 min and washed twice with ice cold 1x PBS at 1500rpm for 5 min. The cell pellet was re-suspended in 3ml Buffer B for every $10x10^6$ cells, for cell lysis, centrifuged at 1500rpm for 5 min and the supernatant was discarded. The cell pellet was then diluted in 300µl sonication buffer, Buffer D supplemented with 1x Complete Protease inhibitor Cocktail per $20x10^6$ cells and kept on ice until sonication. Both Buffer B and D are SDS-based buffers provided by the Shearing ChIP kit. The cells were then sonicated using the Bioruptor Pico sonicator for 20 cycles of 30 sec ON and 30 sec OFF, to achieve sonication of chromatin to DNA fragment sizes ranging between 200 and 500 base pairs. The sonicated chromatin was then centrifuged at 12000rpm for 10 min to remove any cell debris and stored at -80°C in aliquots.

2.12.4. Immunoprecipitation

Chromatin was centrifuged at 13000 rpm for 10 min at 4°C to remove any remaining cell debris. 25µL per 300µl of chromatin was stored at -20˚C as Input control. Pre-

clearing of chromatin prior to binding to the primary antibody was done using 60µl BSA blocked Protein A Agarose/Salmon sperm DNA beads for 30 minutes at 4°C. The supernatant was collected and was incubated with 10µg of the antibody (Table 7) and 10µg of the corresponding IgG overnight at 4°C on a rotator wheel. All the beads were blocked with 10mg/ml BSA for 45 minutes at room temperature. The following day, chromatin was incubated with 75µl blocked Protein A Agarose/Salmon sperm DNA beads for 1 hr at 4°C on a rotator wheel. The beads were then washed sequentially once with 1mL of Low salt, once with 1ml High salt, once with 1ml LiCl and twice with 1ml TE wash buffers for 3 min each at 4°C. The beads were recovered with 30sec centrifugation at 2000rpm at 4°C. Bound chromatin was eluted by incubating the beads in 250µl Elution buffer for 15 min at room temperature. This was repeated twice with the total volume of eluted chromatin reaching 500µl. Input was also re-suspended in Elution buffer up to a final volume of 500µl.

2.12.5. De-cross Linking and Purification of chromatin

20µl 5M NaCl was used for chromatin de-cross linking at 65°C overnight. The decrosslinked samples were deproteinised with 10mg/ml Proteinase K, 0.5M EDTA and 20µl Tris-HCL pH 6.5 for 1 hr at 45°C. The DNA was recovered by phenol/chroloform extraction followed by isopropanol precipitation and washed with 75% ethanol with 1µl 20µg/ml Glycogen as carrier. The DNA was diluted into 40µl HPLC grade water for all samples apart from the Input samples that were diluted in 160µl HPLC grade water.

2.12.6. Gel electrophoresis

Gel electrophoresis was used to assess the chromatin shearing efficiency of sonication. 10µl of the sonicated chromatin was de-cross linked and the DNA was purified as described in section 2.12.5. Half of the DNA was then loaded onto a 1.5% agarose gel and run at 95V for 40-50 min.

2.12.7. Real-time PCR with SYBR green

Real-time PCR was carried out in the 7900HT Real-time PCR machine using SYBR green master mix. Primers (Table 8) were synthesised by Metabion GmHB (Metabion, Germany). 1µl of each de-cross linked sample was used as a template for the reaction with 1x SYBR green mix as described in section 2.10.2.

Enrichment for a specific DNA sequence was calculated using the comparative C_T method where target quantities were normalised relative to the PCBP2 gene. Data collection was analysed and plotted using Microsoft Excel. The fold difference of a given target sequence precipitated by a specific antibody was determined by dividing the amount of target sequence in the immunoprecipitation (IP) fraction by the amount of target sequence in input DNA. Therefore, for each reaction, the ratio of IP to input DNA is calculated by subtracting the C_T determined for the target sequence of the IP samples from the C_T determined for the target sequence of the reference sample and taking the resulting value to the power of 0.5.

Table 7, Antibodies used in Chromatin immunoprecipitation experiments

Antibody	Concentration	Supplier	Catalogue No
Dimethyl Histone H3 (Lys4)	10µg	Millipore, GaA, Darmstadt Germany	07-030
MeCP2	10µg/ChIP	Abcam, Cambridge UK	Ab2828

Table 8, Primer sequences used in real-time PCR following ChIP

Gene	Forward primer (5'-3')	Reverse Primer (5'-3')	Product
γ-promoter	ACATTTCACAATCCCTGAAC	TAGCCTTTGCCTTGTTCCG	206
β-promoter	CAATTTGTACTGATGGTATGG	GGTGTCTGTTTGAGGTTGC	280
ε-promoter	CACAAACTTAGTGTCCATCCATCAC	CCCTGTTCTCCATGGTACTTAAAA	111
δ-promoter	ATCTCAGGGCAAGTTAAGGG	GTAAGCAACAGTCGACTCTG	152
Intergenic ε-γ	TCCCACTCTGTGGGTTGTCTGTTT	CCCTTCTACACATTGGCTTAGGAAAGG	158
Cyclin D1	TGCCACACACCAGTGACTTT	ACAGCCAGAAGCTCCAAAAA	60
HS1	CAAGCGTGGGGACTGAGAAGG	TGCTGAGCTGTGATG	85
HS2	GCTTACAGGGCAGATGGCAAAA	GATGCCGTTTGAGGTGGAGTTTTA	48
HS3	TAGGTGGTTAGGTCAGGTTGGTGG	AATGCTGCTATGCTGTGCCTCC	122
HS4	TGGCATCTAGCGCAATGACTT	GGGCAAGCCATCTCATAGCTG	194
HS5	ATTTCTTTCCCCACACCTCCTTCC	TGAGAAATGAGAGAGAGAATGGGGAG	83

2.13. Proteomics

2.13.1. Materials

	Description	Supplier
Kit	iTRAQ reagent – 8plex box	AB Sciex, MA USA
Sonicator	Vibra cell sonicator	Sonics & Materials Inc, Newtown, USA
Reagent	Bradford reagent	BioRad laboratories Inc, CA USA
Reagent	Trypsin	Roche Diagnostics, Basel Switzerland
Reagent	Ultrapure water	Sigma Aldrich Co, St Louis USA
Reagent	Isopropanol	Sigma Aldrich Co, St Louis USA
Concentrator	Savant SpeedVac kit ISS1110	Thermo Scientific, MA USA
Column	150 x 4.6mm, 3m5µm XBridge C18 column	Water SAS, Saint-Quentin En Yvelines Cedex, France
Mass Spec	nano-ESI Orbitrap-XL mass spectrometer	Thermo Scientific, MA USA
Reagent	Acetonitrile	Sigma Aldrich Co, St Louis USA
Reagent	Ammonium hydroxide	Sigma Aldrich Co, St Louis USA
Column	the Acclaim PepMap 100, an 100µm x 2cm C18, 5µm 100 Å trapping column	Thermo Scientific, MA USA
Column	Acclaim PepMap RSLC	Thermo Scientific, MA USA
Reagent	Formic acid	Sigma Aldrich Co, St Louis USA

Mobile Phase composition used for High-pH Reverse Phase (RP) Peptide Fractionation

	Acetonitrile (v/v)	Ammonium hydroxide (v/v)
Phase A	2%	0.05%
Phase B	100%	0.05%

Mobile Phase composition used for Liquid chromatography–Mass spectroscopy analysis

	Acetonitrile (v/v)	Formic acid (v/v)
Phase A	2%	0.1%
Phase B	100%	0.1%

2.13.2. Introduction

Whole cell lysates of primary human erythroid cultures before and after treatment with 300nM decitabine underwent peptide quantitation based on the iTRAQ label

quantitation method. Isobaric tags for relative and absolute quantitation (iTRAQ) provide a non-gel based multiplex protein quantitation technique for identification of differentially expressed peptides/proteins as a function of different biological conditions including disease or drug-treated states. As the name suggests the iTRAQ technology utilises isobaric reagents to label the primary amines of peptides and proteins. The labelling reagent (Figure 11) consists of an N-methylpiperazine reporter group that is unique to each iTRAQ reagent, a neutral balance group and an N-hydroxyl succinimide ester group that reacts with the N-terminal amino groups of peptides. The tags are designed so that on analysis of the labelled samples by collision-induced dissociation (CID), the label is released to give rise to a reporter ion with a specific mass-to-charge ratio (Thompson *et al.*, 2003). The balance groups present in each of the iTRAQ reagents function to make the labelled peptides from each sample isobaric (i.e. to have the same mass). iTRAQ is one of the methods that allows the comparison of proteins between experiments as isobaric labelled proteins can co-migrate in liquid chromatography acting as a more precise reciprocal internal standard. All the tags have the same overall mass and are distinguished only upon fragmentation by MS (Thompson *et al.*, 2003) (Figure 12). This allows the simultaneous determination of both identity and relative abundance of peptides in tandem-mass spectra. Moreover, iTRAQ allows the multiplex quantitation of proteins in up to eight different samples which is useful in comparing different parameters within the same experiment, while eliminating any biases due to the experimental procedures.

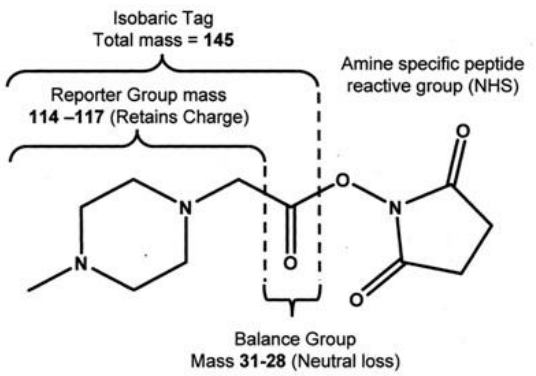


Figure 11, Structure of the iTRAQ reagent consisting of a reporter group, a balance group and an amine specific peptide reactive group. The reporter group ranges in mass from m/z 114.1 to 117.1, while the balance group ranges in mass from 28 to 31Da, such that the combined mass remains constant (145.1Da) for each of the reagents. *Ross P L et al. Mol Cell Proteomics 2004;3:1154-1169*

iTRAQ workflow (Figure 13) outlines the preparation of peptide samples for analysis. Samples to be quantified are initially lysed to extract proteins. After using a standard protein assay to estimate the protein concentration of each sample, proteins are treated for bisulphide bond reduction and block of cysteine residues in proteins. Reduced and blocked proteins are then digested using an enzyme, such as trypsin, to generated proteolytic peptides and are labelled with a different iTRAQ reagent. Once labelled, the different labelled digests are combined into one sample mixture and analyzed by LC-MS/MS for both identification and quantitation. The sequence of a peptide is determined by the production of ions that are generated from cleavage of peptide interresidue bonds. Relative quantitation of iTRAQ-tagged peptide in each sample is facilitated through analysis of the intensity of the reporter groups that are generated upon fragmentation in the mass spectrometer. The experiments were performed by Dr Konstantinos Vougas at the Academy of Athens under the supervision of Dr Elena Katsantoni. The list of proteins generated from the proteomic experiments was then given to me for data analysis.

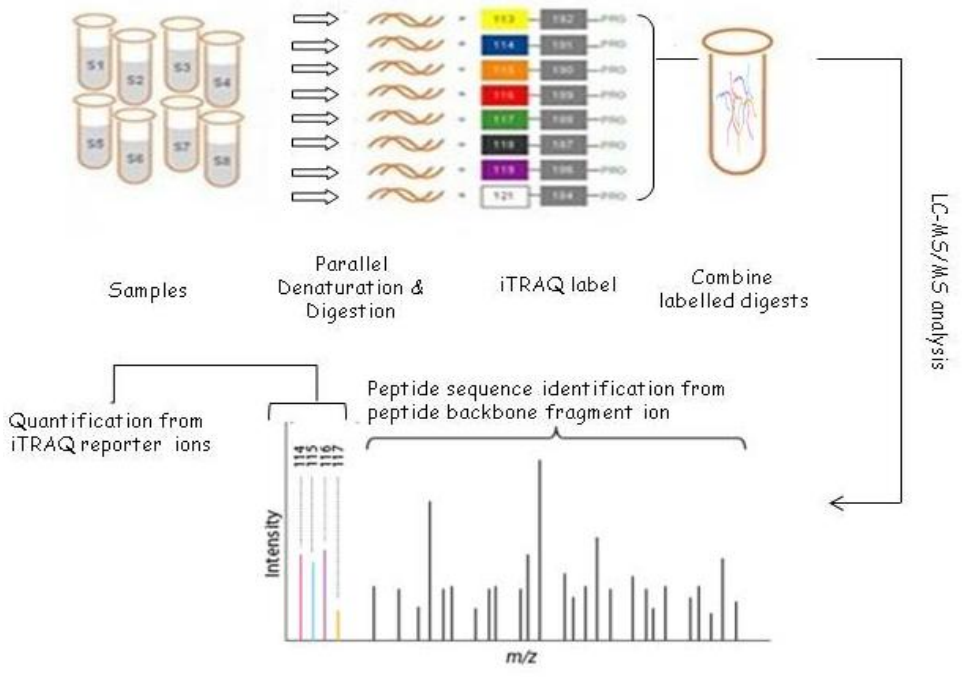


Figure 12, Outline of the procedure followed by quantitative iTRAQ proteomic approach. Adapted from *http://www.ias.ac.in/meetings/myrmeet/21mym_talks/rmadhubala/img39.html* and *http://www.mrmproteomics.com/itraq-protein-quantification/*

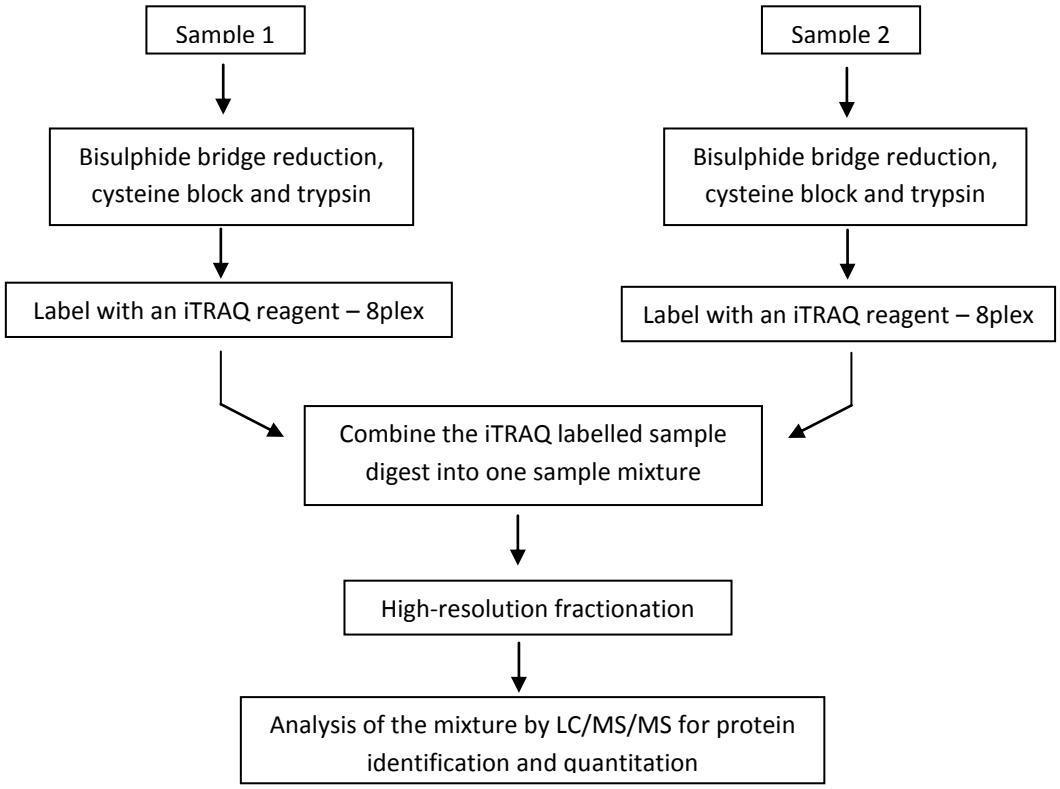


Figure 13, Workflow of the sample preparation, labelling and analysis followed in the quantitative iTRAQ proteomic approach

2.13.3. Samples dissolution and protein quantitation

Frozen cell pellets corresponding to each sample were re-suspended in 200-300µl Dissolution Buffer containing 0.5M Triethylammonium bicarbonate (TEAB) and 0.05% SDS (provided by the iTRAQ kit) for every $4x10^6$ cells. Effective pellet disruption and homogenisation were ensured by pulse probe sonication at an amplitude of 29% with pulse ON for 0.1sec and pulse OFF for 0.2sec for a total sonication time of 0.5-1 min. Non-homogenised cellular debris was separated from the protein solution with centrifugation at 13000rpm for 10min. The amount of protein in each sample was measured with the Bradford protein assay as described in Section 2.11.3. For each sample, a total of 100µg protein was then diluted with dissolution buffer to a final volume of 20µl.

2.13.4. Reduction of disulfide bonds and Blocking cysteines

Cysteine disulfide bonds were reduced with tris(2-carboxyethyl)phosphine (TCEP) using 2µl 50mM TCEP for every 20µl sample, followed by incubation at 60°C for an

hour. Cysteine residues were blocked by the addition of 1µl 200mM Methyl methanethiosulfonate (MMTS) in isopropanol for 10min at room temperature.

2.13.5. Protein digestion and iTRAQ labelling

Samples were then diluted with 14µl ultrapure water and 6µl of 500ng/µl proteomics grade trypsin solution resulting in an enzyme:substrate ratio of 30:1 (v/v) following which they were incubated with trypsin overnight at room temperature. After tryptic digestion, the peptide samples were labelled with isobaric tags. For isobaric labelling, each label (8µl) was diluted in a total volume of 100µl with isopropanol. Once the labels were ready, one label was added to each digested sample and incubated at room temperature for two hours. The 8 different labelled samples were then pooled and vacuum dried overnight using the Savant speedvac concentrator.

2.13.6. High-pH Reverse Phase (RP) Peptide Fractionation

High-pH RP C18 fractionation of the iTRAQ 8-plex labelled peptides was performed on the Dionex P680 pump coupled with the PDA-100 photodiode array detector using the 150 x 4.6mm, 3m5µm XBridge C18 column. Peptide pellets were dissolved in 200µl mobile phase A by bath sonication. Samples were centrifuged at 13000rpm for 5min and the supernatant was injected into the column through a 200µl sample loop. The peptide separation was performed as follows: 10 min isocratic 5% phase B, 50min gradient up to 50% of phase B, 10min gradient up to 70% of phase B, followed by 10min gradient up to 95% phase B at a flow rate of 0.4ml/min. The signal was monitored at 280, 254 and 215nm while maintaining a temperature of 30˚C. Peptide fractions were collected every minute and were finally dried with speedvac concentrator for 4-5 hours and stored at -20˚C before being analysed by LC-MS.

2.13.7. Liquid chromatography–Mass spectroscopy (LC-MS) analysis

All LC-MS experiments were performed on the Dionex Ultimate 3000 UHPL system coupled with the high resolution nano-electrospray ionization (ESI) Orbitrap-XL mass

spectrometer. Individual high-pH RP peptide fractions were reconstituted in 30µl loading solution composed of 2% acetonitrile and 0.1% formic acid. 2µl sample volume was injected and loaded for 8 min onto the Acclaim PepMap 100, an 100µm x 2cm C18, 5µm 100 Å trapping column, coupled with the ulPickUP Injection mode and the loading pump operating at 5µl/min flow rate. For peptide separation the Acclaim PepMap RSLC, a 75µm x 25cm nanoViper C18, 2µm, 100Å column retrofitted to a PicoTip emitter (FS360-20-10-D-20-C7) was used for multi-step gradient elution. The gradient elution method was as follows: gradient up of phase B to 40% for 80 min and up to 85% for the next 5 min, followed by 5 min of isocratic 85% phase B, a drop of phase B to 3% in 2 min and finally 8 min isocratic equilibration at 3% of phase B. The flow rate was maintained at 300nL/min and the column temperature at 35˚C. Gaseous phase transition of the separated peptides was achieved with positive ion electrospray ionization by applying a voltage of 2.5kV. For every Mass Spectrometry (MS) survey scan, the top 10 most abundant multiply-charged precursor ions between mass/charge (m/z) ratio 350 and 1900 and intensity threshold 500 counts were selected with FT mass resolution of 15,000 and subjected to high-energy collisional dissociation (HCD) fragmentation with an isolation window of 1.2Da. Tandem mass spectra were acquired with FT resolution of 7500 within m/z range of 100-1900. The normalised collision energy was set to 45 and already targeted precursors were dynamically excluded for further isolation and activation for 45sec with 5ppm mass tolerance.

2.13.8. Database search

The HCD tandem mass spectra collected from RP fractions were analysed through the Proteome Discovered software (v1.4) for peptide and protein identifications. All spectra were searched against a UniProt Fasta file containing 20,200 human reviewed entries. The search parameters included Precursor Mass Tolerance of 10ppm, Fragment Mass Tolerance of 0.05Da, Dynamic Modifications were Oxidation of M (+15.995Da), Deamidation of N, Q (+0.984Da), Phosphorylation of S,T,Y (+79.966Da) and Static Modifications at any N-terminus, K Y (+304.205Da) and Methylthio at C (+45.988Da). iTRAQ quantitation was also performed through the same software. Protein ratios were normalised to protein median and peptides with missing iTRAQ channels were excluded from relative protein quantitation.

2.13.9. Data Analysis

Normalization and quantitation of the proteins led to the generation of differentially expressed proteins, where the expression levels of each protein and their intensity was calculated. The final list of proteins was an average of the protein ratios of all three experiments and included proteins that were expressed in all three experiments. Functional annotation analysis of differentially expressed proteins was carried out using two bioinformatic resources, the Database of Annotation, Visualization and Integrated Discovery (DAVID) (Huang da *et al*., 2009) and GeneCodis (Carmona-Saez *et al*., 2007, Nogales-Cadenas *et al*., 2009, Tabas-Madrid *et al*., 2012). Both resources use GO terms to annotate the proteins into functional groups.

2.14. Lentivirus shRNA-mediated knock-down

2.14.1. Materials

	Description	Supplier
Buffer	NEB CutSmart buffer	New England Biolab, MA USA
Media	Luria Broth	Invitrogen Inc, Paisley UK
Antibiotic	Ampicillin	Sigma Aldrich, St Louis USA
Broth	Agar Granulate	Melford, Sulffock UK
Enzyme	StuI (10000U/ml)	New England Biolab, MA USA
Kit	NucleoSpin Plasmid DNA purification	Macherey-Nagel GmbH, Duren Germany
Reagent	Isopropanol	Sigma Aldrich, St Louis USA
Reagent	EMSURE ethanol	Merck, Darmstadt Germany
Reagent	Sodium Acetate	Sigma Aldrich, St Louis USA
Kit	NucleoBond Xtra Maxi Columns	Macherey-Nagel GmbH, Duren
Media	DMEM	GIBCO Invitrogen Inc, Paisley UK
Supplement	FBS	GIBCO Invitrogen Inc, Paisley UK
Reagent	Polyethylenimine	Polysciences GmbH, Eppelheim, Germany
Media	RPMI	GIBCO Invitrogen Inc, Paisley UK
Instrument	SW28 rotor	Bechman Coulter Inc, California USA

Buffer 1:

Material	Final concentration
Tris	50mM
EDTA	10mM

Buffer 2:

Material	Final concentration
NaOH	0.2M
SDS	1%

KAc:

Material	Final concentration
KAc	3M
Glacial Acetic Acid	5M

HBS solution:

Material	Final concentration	Supplier
NaCl	280mM	Merck KGaA, Darmstadt Germany
HEPES	50mM	Scharlau S.L., Mas d'En Cisa Spain
$Na_2HPO_4.7H_2O$ (pH7.05)	1.42mM	Scharlau S.L., Mas d'En Cisa Spain

2.14.2. Introduction

RNA interference (RNAi) is an evolutionarily conserved mechanism of post-transcriptional gene silencing induced by introducing the double-stranded RNAs into cells. Several methods of RNA interference (RNAi) have evolved over time, among them are the widely used exogenously synthetic and endogenously expressed small interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs). Although the simplest approach involves the transduction of chemically synthesised siRNAs oligonucleotides directly into the cytosol, siRNA-mediated RNAi are limited to only cells capable of transduction due to the variable transduction efficiencies of different cell types (Moore *et al.*, 2010). The shRNAs are more efficient than siRNAs on the induction of gene silencing and currently have evolved as an extremely powerful and the most popular gene silencing reagent (Cheng and Chang, 2007). shRNAs are synthesised within the cell by DNA vector-mediated production. They can be transduced as plasmid vectors encoding shRNAs transcribed by RNA pol III or modified pol II promoters, or through infection of the cell with virally produced vectors. The shRNA sequence consists of two complementary 19-22bp RNA sequences linked by a short loop of 4-11 nucleotide similar to the hairpin found in naturally occurring miRNA. Following transcription, the shRNA sequence is exported to the cytosol where it is recognised by an endogenous

enzyme, Dicer, which processes the shRNA into the siRNA duplexes which are able to bind to the target mRNA and is thus incorporated into the RISC complex for target-specific mRNA degradation. There are multiple methods of introducing siRNA and shRNA into cells depending on whether transient or stable expression is desired as well as on the model system. Lentiviral-mediated transduction provides a convenient method of introducing shRNA into dividing or non-dividing cells and in general is less toxic to the cells than adenoviral-mediated transduction (Moore *et al.*, 2010).

2.14.3. Experimental Design

Lentiviral-mediated transduction of shRNAs was used in the current study to knock-down the mRNA levels of 17 genes identified by proteomic analysis of primary human erythroid cultures as potential genes involved in the reactivation of γ-globin expression following treatment by decitabine. shRNA-containing vectors were obtained from the TRC Mission human and mouse library from Sigma in the form of glycerol stocks. Packing was done as described by the Sigma Mission library protocol. shRNAs were cloned into pLK0.1 vector using EcoRI and AgeI and verified by sequencing. The pLK0.1-TRC cloning vector contains a stuffer that is released upon digestion with EcoRI and AgeI. The oligos contain the shRNA sequence flanked by sequences that are compatible with the sticky ends of EcoRI and AgeI. The forward and reverse primers of each shRNA are annealed and ligated into the PLK0.1 vector, producing a final plasmid that expresses the shRNA of interest (Figure 14).

Glycerol stocks were streaked onto LB agar plates for generation of single colonies. Isolated single cell derived colonies for each construct were then used as source inoculums for miniprep preparation. The plasmid DNA was isolated from the miniprep preparation and was digested with StuI restriction enzyme to check for the presence of shRNA. The remaining miniprep was used for preparation of Midi and Maxi preps for generation of large quantities of plasmid DNA, necessary for the production of lentiviral particles containing the shRNA vector. Lentivirus was produced by transient transduction of 293T cells and was further used for transduction in primary human erythroid progenitor cells in the presence and absence of the drug. The efficiency of each shRNA was determined by quantitative Real-time PCR. The effect of each shRNA

on γ-globin expression was determined by quantitative real-time PCR and Western blot analysis.

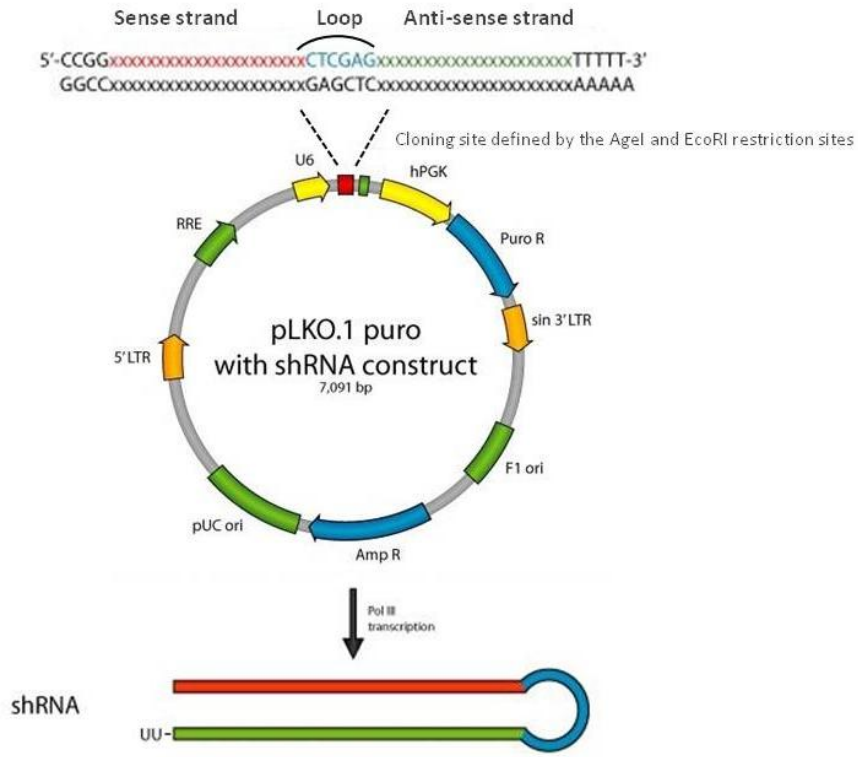


Figure 14, Map of the pLKO.1 vector containing an shRNA insert. The pLK0.1-TRC cloning vector contains a stuffer that is released upon digestion with EcoRI and AgeI. shRNAs, consisting of two complementary 19-22bp RNA sequences linked by a short loop of 4-11 nucleotides, are then cloned into pLK0.1 vector using EcoRI and AgeI sticky ends. The forward and reverse primers of each shRNA are annealed and ligated into the pLK0.1 vector, producing a final plasmid that expresses the shRNA of interest upon transcription by Pol II.

2.14.4. Single colony generation from glycerol stock and preparation of bacterial miniprep cultures

Glycerol stocks harbouring sequenced verified shRNA cloned into the pLKO.1 vectors, were obtained from the TRC Mission Sigma library (Appendix II). Five glycerol stocks corresponding to clones for five different shRNA constructs were purchased for each of the 17 genes under investigation, each clone targeting different regions of the gene sequence. The glycerol stocks were streaked on LB agar plates containing 10μg/ml Ampicillin for generation of single cell derived colonies. Glycerol stocks were diluted

with 10µl LB medium containing 1x ampicillin. Using a sterile pipette, 10µl of the diluted glycerol stocks were streaked onto LB agar plates and incubated overnight at 37˚C.

Isolated single colonies for each construct was then used as a source inoculum for 5ml miniprep preparation where each colony was grown in LB medium containing 10µg/ml ampicillin at 37˚C overnight.

2.14.5. Extraction of miniprep plasmid DNA and screening for inserts

Plasmid DNA was extracted from minipreps by re-suspension of bacterial pellets derived from 1.5ml miniprep cultures into 250µl Buffer 1. 250µl of Buffer 2 were added to the re-suspended bacteria, followed by 250µl of 3M KAC. The solution was then centrifuged for 10min at 13000rpm at room temperature and the pellet was re-suspended in 0.7x volume isopropanol. After centrifugation at 13000rpm for 10 min, the pellet was washed with 70% ethanol and the air-dried DNA was dissolved in 40µl TE supplemented with 40µg/µl RNase. 2µg of the plasmid DNA was screen for the presence of inserts by restriction enzyme digestion with StutI for 30min at 37°C. The digested DNA was separated on 1% agarose gel resulting in two fragments of 995bp and 6.08kb sizes.

2.14.6. Midi preparation and DNA purification

The remaining miniprep was grown into 100ml LB supplemented with 10µg/ml ampicillin at 37°C overnight with shaking. The bacterial cell culture was centrifuged at 4500xg for 20min at 4 °C. The cell pellet was re-suspended in 8ml Buffer 1 and lysed with the addition of 8ml Buffer 2 and mixed well by inversion. For neutralization, 8ml KAC was added to the lysate and mixed well by inversion. The lysate was then centrifuged for 30 min at 4°C and the supernatant was collected. 0.7 x volumes of isopropanol was added to the supernatant, mixed and then centrifuged at 4000rpm for 20min at 4°C. The pellet was then air dried and re-suspended into 200µl TE buffer supplemented with 20µg/ml RNAse. The plasmid DNA was then purified using the NucleoSpin Plasmid DNA purification kit according to the manufacturer’s instructions.

Two volumes of Buffer A3 was added to the DNA solution and mixed well by inversion. The mixture was then loaded onto a NucleoSpin Plasmid column and centrifuged at 11000xg for 1 min. The supernatant was discarded and the column washed with 600µl Buffer A4. The column was centrifuged at 11000xg for 1min and supernatant was discarded. In order to remove excess ethanol wash buffer from the column, the column was centrifuged for 2 mins at 11000xg. The DNA was then eluted using 50µl of Buffer AE. For maximum yield, the column was incubated for 1 min at room temperature and then centrifuged at 11000xg for 1 min. The DNA concentration was determined by NanoDrop spectrophotometer as noted in Section 2.8.4. The plasmid DNA was once more tested for the presence of the insert by restriction enzyme digestion as in Section 2.14.5.

2.14.7. Maxi preparation and DNA purification

For generation of large amounts of plasmid DNA, in particular for scramble plasmid vector and psPAX2 packing plasmid, maxi prep amounts of cultures were produced. Plasmid DNA purification was carried out using the Plasmid DNA purification NucleoBond Xtra Maxi kit according to the manufacturer's instructions. Glycerol stocks were used as a source of inoculums for preparation of a 400ml bacterial culture in LB medium supplemented with 10µg/ml ampicillin at 37°C overnight. Bacterial cell culture was centrifuged at 4500xg for 20min at 4 °C. The cell pellet was re-suspended with 12ml Resuspension buffer and lysed with the addition of 12ml Lysis buffer followed by 12ml Neutralization buffer. The column filter was equilibrated with 25ml Equilibration buffer before being loaded with the lysate. The filter was washed with 15ml equilibration buffer and at this point the first filter was removed. The second filter was then washed with 25ml Wash buffer (containing ethanol) and the DNA eluted with 15ml Elution buffer and purified using 0.7 x volume of isopropanol followed by 100% ethanol precipitation and 0.1x volume of Sodium Acetate. The DNA was washed once with 70% ethanol at full speed for 5min at 4°C on a benchtop centrifuge and the DNA pellet re-suspended in 200-400µl H_2O. The DNA concentration was determined by NanoDrop spectrophotometer as noted in Section 2.8.4.

The scramble Plasmid DNA was tested by restriction enzyme digestion with StuI as described in 2.14.5. The psPAX2 packing plasmid DNA was tested by BamHI, EcoRI

and XmnI restriction enzymes. The fragment sizes expected following digestion with the above enzymes are shown in Table 9.

Table 9, Restriction enzyme digestion of psPAX2 packing plasmid with BamHI, EcoRI and XmnI. The table lists the fragment sizes expected following digestion with the above enzymes

BamHI	***EcoRI***	***XmnI***
9373bp	6329bp	4902bp
1007bp	4374bp	2151bp
337bp		1805bp
		1411bp
		434bp

2.14.8. Lentiviral production of plasmid DNA

Lentivirus was produced by transient transduction of 293T cells. A day before viral transduction, 293T cells were cultured in 10cm plates to 30% confluence in DMEM supplemented with 10% foetal bovine serum. On the day of transduction, once the cells have reached ~80% confluence, the medium was discarded and replaced by 6ml DMEM supplemented with 1% FBS. For vector preparation, 5µg of Env plasmid (pMD2_VSVG), 15µg of packing plasmid (psPAX2) and 20µg of transfer vector (pLK0.1/TRC clone) were added to 1ml filtered sterilised 1xHBS. 100µg Polyethylenimine (PEI) diluted in 1ml HBS were added in the DNA/HBS solution in a slow dropwise manner and incubated at room temperature for 15-20min. The virus/PEI precipitate was added in a slow dropwise manner. After 3-4 hours, the medium was replaced with 8ml DMEM supplemented with 10% FCS and incubated at 37°C. The culture medium containing the virus was harvested on the first three consecutive days of transduction by aspiration and stored at 4°C and was replaced by 8ml of fresh DMEM supplemented with 10% FBS. The 24ml viral supernatant was filter sterilised through 0.45µm filter units and then ultra-centrifuged for 4 hours at 20000rpm at 4°C using an SW28 rotor. The virus was re-suspended in 100µl PBS, aliquoted and stored at -80°C.

2.14.9. Viral Transduction of primary human erythroid progenitor cells

Primary human erythroid cultures from healthy donors were transduced on Day 4 of Phase II in 24-well plates. $1x10^6$ cells were seeded in each well in 500ml Phase II media (Section 2.4.2). Each well was transduced by the addition of 25µl virus and incubated for 8 days. Four wells were transduced with the same virus. The cells from two of the wells were used for western blot analysis and the cells from the remaining two wells for RNA extraction. On Day 5 of Phase II, another 500µl Phase II media were added to each well. On Day 6 of Phase II, two wells of each virus were incubated with 300nM decitabine and incubated for 6 days. Four wells were transduced with virus containing the sramble construct vector and four wells were incubated without any virus as negative controls.

On Day 12 of Phase II, the cells were collected and washed once with PBS. Whole cell lysates were prepared from two wells transduced with each virus, one with and one without the agent, with 8µl RIPA buffer (Section 2.12.1) for every $1x10^6$ cells. Equal volumes of sample buffer (Section 2.12.1) were added to the samples and stored at -20°C. Half of the sample (~16µl) was loaded onto an SDS-PAGE gel and were quantified for HBG and GAPDH proteins by western blotting as described in Section 2.12.4.

The two wells destined for RNA extraction, one with and one without the agent, were diluted in 1ml TriFast solution for RNA extraction as described in Section 2.8.3. The RNA concentration was determined by NanoDrop as described in Section 2.8.4 and 1µg RNA was used for cDNA production as described in Section 2.9.

2.14.10. Quantitative Real-time PCR

Real-time PCR on the ABI 7900HT Fast Real-time PCR System with Fast 96-well block module (Applied Biosystems, New Jersey, USA) coupled with SYBR green technology (Applied Biosystems, New Jersey, USA) was used for determining the level of knock-down of each mRNA with each shRNA construct. All primers were purchased from Metabion (Metabion GmbH, Martinsried, Germany) (Table 10). 37.5ng of cDNA was used as template in a 12.5µl total volume reaction mixture (Table 11) with 1xSYBR Green PCR master mix. Amplification and real-time data acquisition was performed

using the following cycle conditions: initial denaturation at 95˚C for 10 min, followed by 40 cycles of 15s at 95˚C and 1 min at 60˚C. Target quantities were normalised to the endogenous GAPDH gene. Relative quantification was performed according to the comparative C_T calculation method. The relative expression of target gene in the treated samples is expressed relative to the expression of the same gene in un-treated samples as $2^{-\Delta\Delta CT}$.

Table 10, Primer sequences used for quantitation of mRNA levels of genes following knock-down with shRNA constructs in the presence and absence of 300nM decitabine in primary human erythroid cultures.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product size
USP11	TTGAGGGGCGTCTTCCAATC	CACAAGGAACCAGCTTTCGC	245
PPP5C	CGAGAGCATGACCATTGAGGA	TGAGTGTGGTTTCCACGAGC	194
TACC3	GATGCAGTGGTAAAGGCGAC	GGCCTGGTACACAACCTCTT	126
SMARCD3	CAAAGGATTCGGGAGCTGGT	GCTTTTGCTTCATGGGCCTC	136
PSMB10	GTTCCAAGACGGGGTCATTCT	GCAGTAGATTTTGGGGGCGA	109
RCBTB2	AGACACGGGGGAGGTCTAT	CCAAGCATACACTTGGCCTTC	181
TAF9	AGCTCGAGAAGTCTGATCATCG	TCGGAAGGCAAACTCCAACA	163
TMEM19	GTGGCGTTGGCTGTTTTCTG	TCCAACGACTAGCCCTCCTA	106
CHUK	AGCGAGCAGATGACGTATGG	GGCACGCTGTTCCAGAGATT	210
EGLN2	GGGTACGTAAGGCACGTTGA	GACAGTGATGGCGTACCTGG	240
BAZ1B	TGATGGCAACCAGGAGCTTT	TGAAATGACCCGGGCTTCAA	124
HTATIP2	AGGCCTGGAGTTCTGTTATGTG	CAGGTCATGGATGGCCTTGT	204
PYCARD	AGCCAGGCCTGCACTTTAT	GCATCTTGCTTGGGTTGGTG	153
HEXIM1	CCAAGAAGAAGCGGCATTGG	GGAGTACAGGCCGGTTTTGA	206
ARHGA4	GAGGAGCAGGAGGTGTCTTG	TGGCCTGAGCTCTGGATAAAC	134
CHD5	ACATGATCCTCAACGAGCCC	GCCTGCTCCAGCAGCTTAAA	103
S100A8	AAGGGGAATTTCCATGCCGT	GCCACGCCCATCTTTATCAC	176

Table 11, Real time PCR reaction mixture used for quantitation of mRNA levels of genes following knock-down with shRNA constructs

Reagent	Final concentration	Volume (µl)/reaction
SYBR green mastermix (2x)	1x	6.25
Primers (5µM)	0.3µM	0.75
RNase, DNase-free Water	-	3.25µl

3. RESULTS

3.1. Defining the correct parameters for *in vitro* screening in primary human erythroid progenitor cells

3.1.1. Introduction

Major part of the project, including screening of potential agents and delineation of the molecular mechanism of action of an HbF inducer, was performed in primary human erythroid progenitor cell cultures. Initial primary human erythroid cultures set up for the study had a high baseline HbF level in the un-treated mature erythroblasts, and demonstrated differences in the level of response to known agents compared to the literature. In order to ensure that the most optimal conditions were used for the preparation of the primary human erythroid cultures, different parameters were investigated before proceeding with any experiments.

Primary human erythroid progenitor cell cultures are generally thought to be the best available *in vitro* model of human erythopoiesis due to their resemblance with *in vivo* erythropoiesis. For several years the semi-solid erythroid colony technique was the main method of preparation of primary human erythroid progenitor cells. The semi-solid colony technique is suitable for enumeration and study of clonal erythropoiesis. However, immobilization of cells on semi-solid medium results in several disadvantages, including low cell yield and difficulty in manipulation of cells for biochemical, molecular and immunological characterisation. Fibach *et al.* (1989) introduced the first liquid culture system to support erythropoiesis *in vitro*. This system has many advantages over the other experimental models including the use of peripheral blood cells as a source of $CD34^+$ cells and ability of the system to recapitulate many aspects of the *in vivo* erythropoiesis.

Following the introduction of the liquid culture by Fibach *et al.* (1989) (Section 2.4), a large number of methods has emerged for culture of primary human erythroid progenitor cells ranging from single-phase to hanging drop protocols, each providing a different approach for a more precise *in vitro* representation of erythropoiesis. Van den Akker *et al.* (2010) demonstrated that $CD34^-$ peripheral blood mononuclear cells (PBMC) yielded 10-20 times more erythroblasts than $CD34^+$ cells purified by immuno-magnetic bead technology from the same amount of peripheral blood. Sonoda (2008) suggested that peripheral blood has various progenitor types that have the capacity to differentiate into erythroblasts with only a minority being $CD34^+$ cells suggesting that

the environment has a major role in the correct differentiation of progenitors into erythroblasts (Sonoda, 2008). Fibach *et al.* (1989) pointed out the importance of EPO for the maturation of the erythroid-committed progenitors into mature erythrocytes. Dai *et al.* (1991) later showed that SCF was essential for BFUe proliferation and maturation without the need for accessory cells. In contrast, SCF was not necessary for maturation of erythrocytes once they have developed into CFUe-like cells (Dai *et al.*, 1991). The combination of SCF and EPO results in the stimulation of glucocorticoid receptors that promote proliferation of erythroblasts and inhibit erythroid differentiation, while at the same time inhibiting activation of T cell lymphocytes that might be present in the culture (von Lindern *et al.*, 1999).

In addition, globin gene expression patterns, which change during various ontogenic stages, are significantly influenced by culture conditions. Foetal calf serum (FCS) was shown to stimulate γ-globin mRNA and γ-globin production (Constantoulakis *et al.*, 1990). This result in significantly higher HbF levels in erythroid cells cultured in FCS-containing medium than those in the peripheral blood of corresponding donors. Furthermore, Gabbianelli *et al.* (2003) showed that c-kit alone or in combination with dexamethasone induces expansion of effective erythropoiesis and stimulates HbF levels to foetal levels in cultures from thalassaemic patients' peripheral blood.

3.1.2. Characterisation of HbF levels and differentiation pattern in primary human erythroid cultures

Primary human erythroid cultures were set up from buffy coats obtained from the National blood bank in Nicosia. A buffy coat usually originates from 500ml whole blood and is the byproduct of the processing of peripheral blood donation. The buffy coat consists of white blood cells and platelets. By using buffy coats, we ensured that the yield of erythroblasts at the end of the cultures will be in the range of millions, rendering screening of potential agents achievable and efficient.

The progress of erythroid differentiation in Phase II, the erythropoietin-dependent phase that promotes the differentiation of progenitors, was investigated by cytospin preparations (Section 2.5) where the morphology of the different stages of maturation (Figure 15) were monitored along with benzidine staining. As can be seen by the

morphological changes, erythroid progenitors undergo differentiation throughout Phase II, terminating in the orthochromatic normoblast stage by the end of Phase II. On day 3 of Phase II, erythroid blasts predominate in the culture. First signs of maturation are observed on day 5, when the blasts have matured into pre-pro and pro-erythroblasts. From that point onwards, maturation moves much faster onto polychromatic and orthochromatic normoblasts on day 6-10. By day 13, the percentage of differentiated cells has reached an average of 67% of the total cell population as determined by benzidine staining. Cytospins show that maturation of blasts is not always synchronised and homogeneous and that the culture can be sometimes contaminated with other types of cells such as lymphocytes and monocytes. These provide a possible explanation as to why the percentage of benzidine positive cells was below the 90% levels suggested in the literature.

As erythroblasts mature, they accumulate haemoglobin (Figure 16), with gradual increase in HbF levels, reaching a maximum value on day 7 of Phase II. In a similar manner to *in vivo* erythropoiesis, HbF levels dropped as the erythroblasts reached the orthochromatic stage, mimicking the foetal to adult switch. Although the percentage of HbF was observed to decrease at the end of the culture period, the final percentage of HbF observed on day 13 of Phase II (~5%) was higher than the percentages observed in the peripheral blood of healthy donors (1-2%). This may be attributed to factors present within the environment of the culture that can stimulate the expression of HbF.

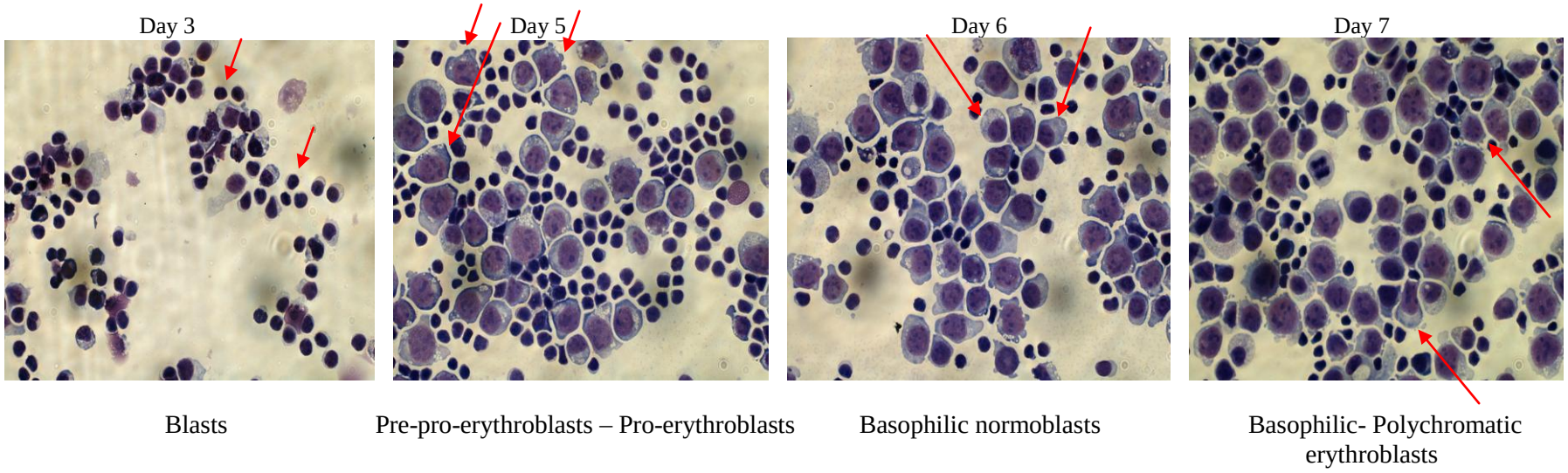


Figure 15, Characterisation of morphological stages of erythropoiesis *in vitro*. Cytospin preparations correspond to day 3, 5, 6, 7, 8 and 13 of Phase II of primary human erythroid progenitor cell cultures from healthy donors. The cytospins were stained with eosin red and methylene blue. Cytospins show how the erythroid blasts (day 3) progress and mature into orthochromatic erythroblasts by day 13 in Phase II of the two phase method by Fibach (1998). Red arrows indicate the cells that correspond to each maturation stage.

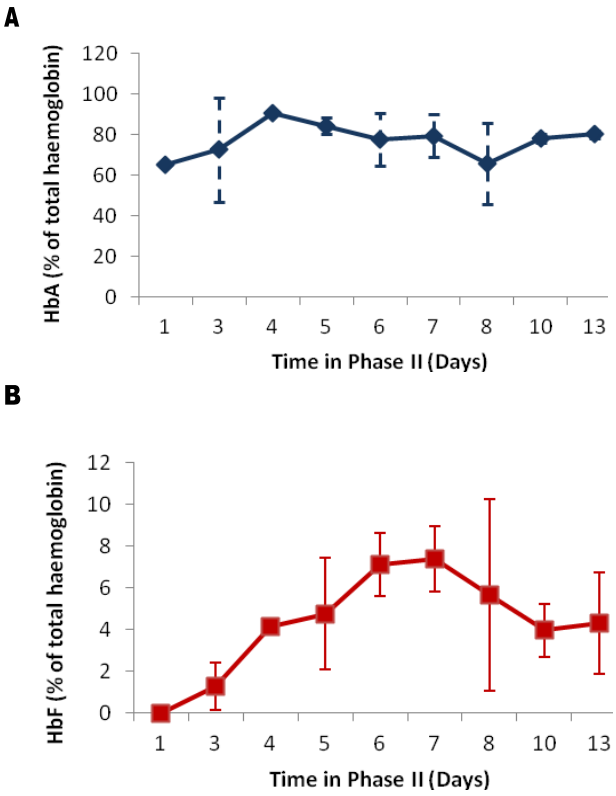


Figure 16, Foetal and adult haemoglobin expression during Phase II of primary human erythroid progenitor cell cultures from healthy donors. HbA (A) and HbF (B) levels were measured on day 1, 3, 4, 5, 6, 7, 8, 10 and 13 of Phase II by cation exchange chromatography. HbF can be detected from day 3 of phase II, peaks on day 7 of Phase II and drops as erythroblasts mature. The HbF and HbA levels are presented as percentages of the total haemoglobin present in the cells. The results represent the average of 5 experiments with error bars corresponding to the standard deviation.

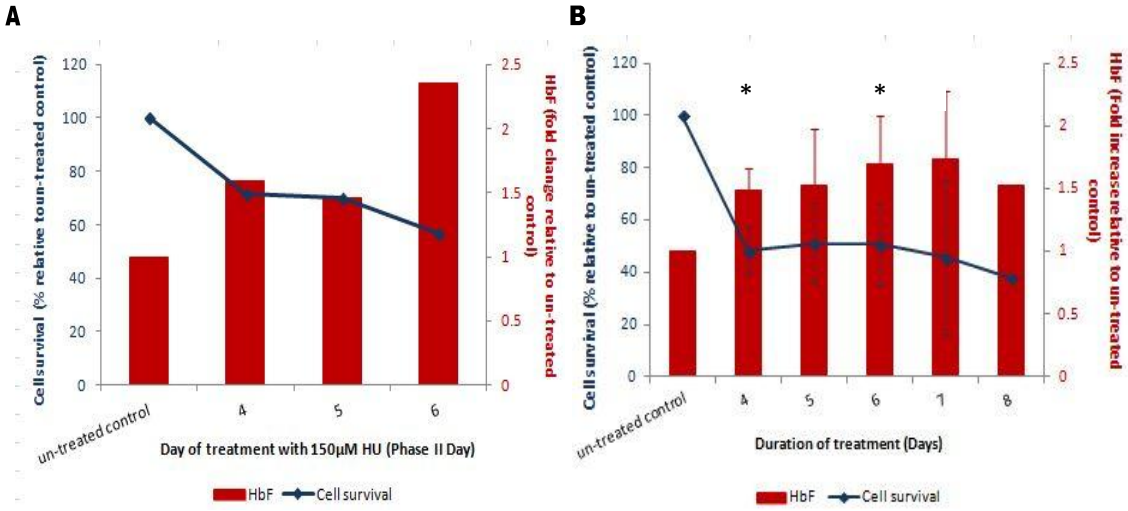


Figure 17, Investigation of the time (A) and duration (B) of treatment on the expression of HbF in primary human erythroid progenitor cell cultures using hydroxyurea as the inducing agent. The highest average HbF induction was observed when the agent was added on day 6 of Phase II and left in the cultures for 7 days. The results are the average of 1-5 experiments with error bars corresponding to the standard deviation. Paired t-test shows that the effect observed when the cultures were treated for 6 and 4 days is highly significant with p-value 0.05<0.01 (*).

3.1.3. Optimisation of time and duration of treatment of primary human erythroid cultures

To identify the optimal time point and duration of treatment, we treated primary human erythroid progenitor cells from healthy donors with hydroxyurea. Treatment of cultures with 150μM HU on different days of Phase II (Day 4-8) (Figure 17A) showed that the inducing activity was highest when the agent was added on day 6, i.e. once the progenitors had reached the basophilic stage. Furthermore, treatment of primary human erythroid cultures with HU for 4 and 6 days increased the expression of HbF significantly when compared to the un-treated control (p-value<0.05, paired t-test) (Figure 17B). However, there was no significant increase in the level of HbF following 5 or 7 days of treatment with HU. Since the induction observed was higher (~1.7) after 6 days of incubation with the HU than after 4 days (~1.5) with similar cytotoxicity (p-value>0.05, paired t-test), we decided to use a treatment period of 6 days in subsequent experiments to obtain the highest possible effect.

The percentage of HbF in primary human erythroid cultures ranged between 2-14%, levels, which were higher than the percentage of HbF found normally in the peripheral blood of adults (<1%). The high baseline of HbF level in primary human erythroid culture can be a limiting factor in the screening of HbF inducers. To demonstrate considerable activity, an agent needs to increase the percentage of HbF by more than 2-fold, i.e. the agent has the ability to increase the percentage of HbF by 100% above the un-treated control. Such a percentage is not achievable with most inducers and thus the effect of moderate inducers may not be considered as significant, and potential HbF-inducing agents could be missed during screening in primary human erythroid progenitor cell cultures. We, therefore, investigated variable concentrations of EPO to define the concentration in Phase II that will provide good differentiation and progression of the culture without over-stimulating HbF levels. Results from a single experiment (Figure 18) show no major changes in cell survival, benzidine staining, $CD235a^+$ percentage, HbF and HbA levels when cells were grown in 0.5U/ml, 1U/ml or 1.5U/ml of EPO in Phase II.

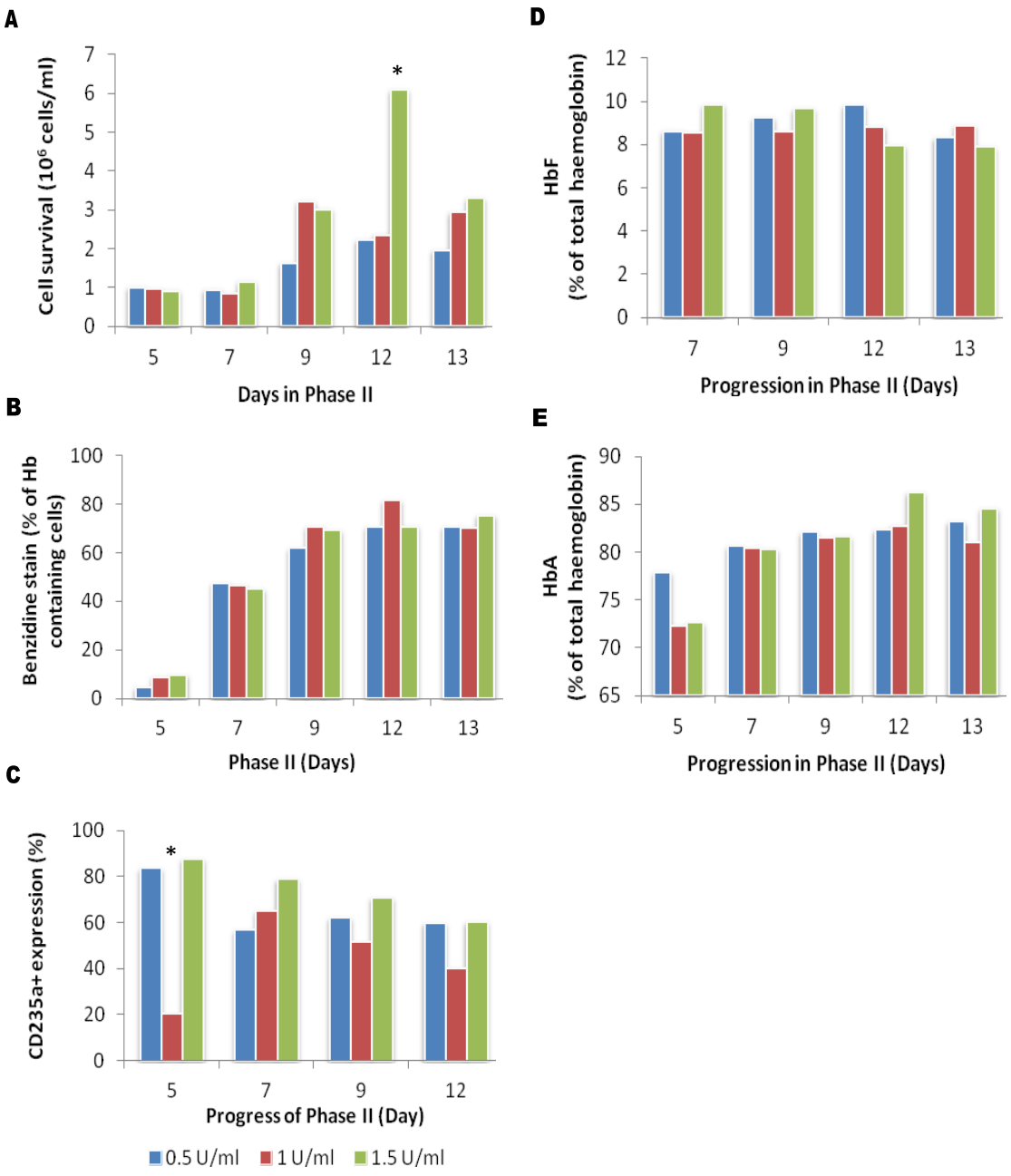


Figure 18, The effect of variable concentrations of EPO on the progression of primary human erythroid progenitor cell cultures during Phase II. Erythroid progenitors were grown in three concentrations of EPO, 0.5U/ml, 1U/ml and 1.5U/ml, in Phase II and the effect on cell survival (A), haemoglobinization (B), differentiation (C), HbF (D) and HbA (E) levels was investigated on days 5, 7, 9, 12 and 13 of Phase II. Similar patterns were observed for all parameters investigated despite the different concentrations of EPO used. The results are from a single experiment. * these results were disregarded given how different they are to the remaining samples.

Constantoulakis *et al.* (1990) suggested that FCS contains factors that possess HbF inducing activity. We thought that this might also be true for the FBS used in the current study, and thus proceeded to investigate whether changes in the percentage of FBS in Phase II will affect the HbF levels at the end of the culture (Figure 19). A reduction in cell numbers was observed when cells were grown in 45% FBS after 3 and 6 days in Phase II, possibly due to inhibition of cell proliferation. No major differences can be observed in the percentage of Hb containing cells between the different percentages of FBS. The percentage of HbF remains around similar levels when cells are grown in 30% or 45% FBS during the first 3 and 6 days of Phase II. At day 12 of Phase II, 30% FBS was shown to stimulate cell survival as seen by the increase in cell numbers but at the same time showing lower HbF levels than 45% FBS. The results are in agreement with the original protocol of the two-phase method by Fibach (1998), suggesting 30% FBS to be most efficient.

Studies have also suggested that foetal sheep serum or charcoal treated FCS results in lower HbF levels in BFUe cultures (Constantoulakis *et al.*, 1990). As a result, we investigated the effect of three types of FBS; a heat-inactivated FBS (FBS 10500), a well characterised and certified FBS with low endotoxin and haemoglobin levels (FBS 16000) and a qualified, EU-approved FBS (FBS 10270) on HbF levels in primary human erythroid cultures from healthy donors (Figure 19) at 30% concentration. FBS16000 promoted cell survival and differentiation as shown by the increase in benzidine positive cells and cell numbers compared to the other two types of FBS. Furthermore, the use of FBS16000 resulted in lower HbF levels through Phase II than any of the two other types of FBS. As a result FBS16000 was chosen as the most suitable FBS to be used in primary human erythroid cultures.

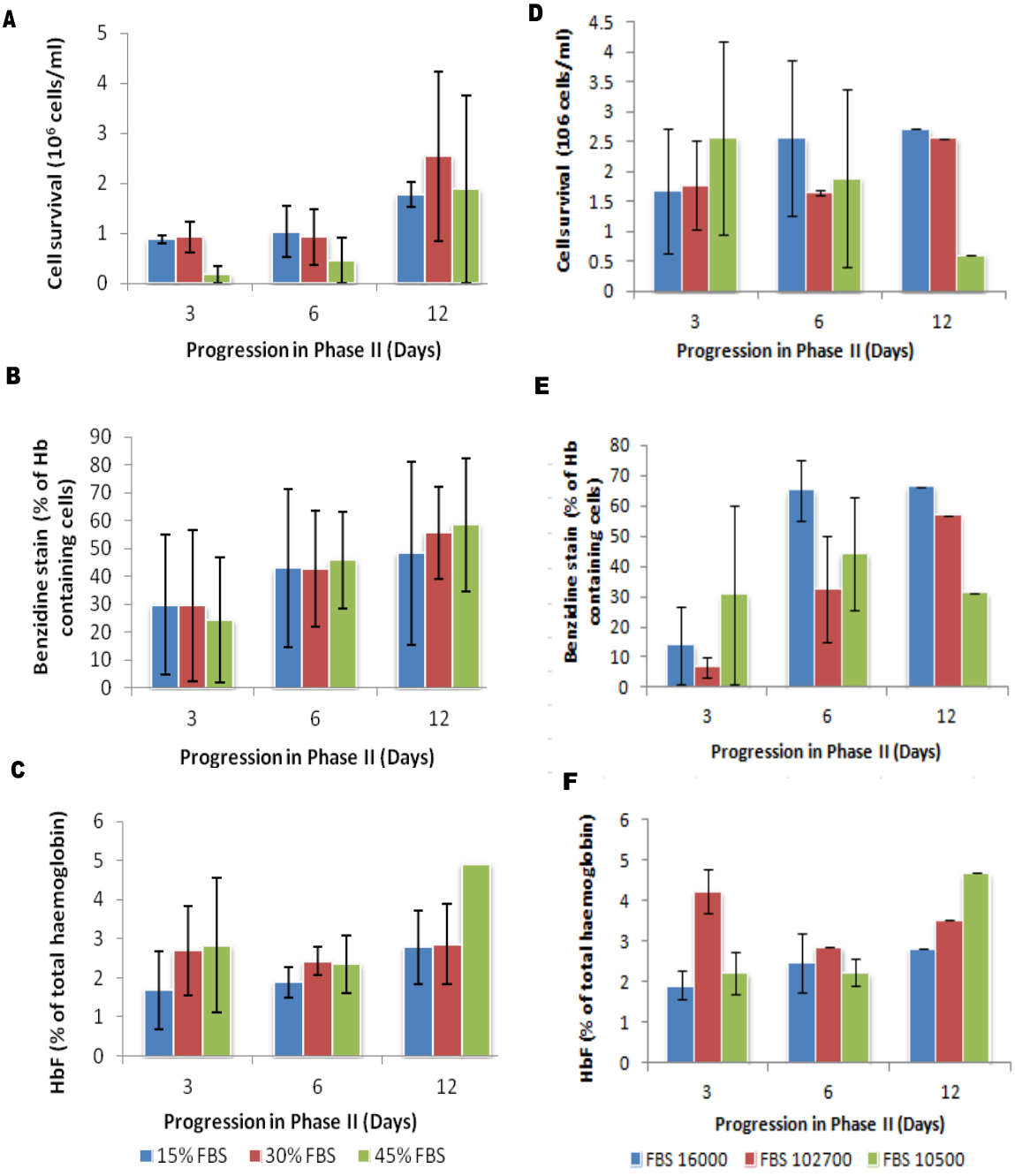


Figure 19, The effect of different FBS parameters on the progression of primary human erythroid progenitor cell cultures. Erythroid progenitors were grown in three concentrations of FBS, 15%, 30% and 45%, (A-C) and in three types of FBS, FBS 16000, FBS 102700 and FBS 10500, at 30% (D-F) in Phase II. The effect on cell survival (A+D), haemoglobinization (B+E) and HbF levels (C+F) was investigated on days 3, 6 and 12 of Phase II. 30% FBS promotes cell survival but limits haemoglobinization and HbF stimulation within median levels between 15 and 45% of FBS (A-C). The results are the average of 2-3 experiments with error bars corresponding to the standard deviation. None of the changes are statistically significant according to the paired t-test (p-value>0.05).

3.1.4. Discussion

Liquid cultures of primary human erythroid progenitor cells is an *in vitro* model that recapitulate many aspects of the *in vivo* erythropoiesis including globin RNA metabolism (Dalyot *et al.*, 1992), cell cycle kinetics (Dalyot *et al*., 1993), expression of cell surface antigens, iron and ferritin metabolism (Vaisman *et al.*, 1997). In contrast to the colony based assay, the liquid method allows easy manipulation of culture conditions at various stages without termination of the cultures, and at the same time improved cell yield per culture (Fibach *et al.*, 1989). Furthermore, cells in suspension cultures undergo morphological changes and haemoglobin synthesis patterns parallel to those in marrow (Wojda *et al.*, 2002). The use of liquid cultures is, however, sometimes limited either by the production of mixed erythroid and myeloid cells or by weak or absent terminal enucleation.

In the current study, we investigated the growth kinetics of the two-phase culture protocol by Fibach using different parameters with respect to their effect on HbF levels. The use of buffy coat as starting material, has led to the generation of a very high cell yield, reaching a total of around $2x10^8$ cells at the end of Phase II. Although the haemoglobin containing erythrocytes in our culture do not reach the 90% level suggested in the literature, the cultures progress correctly towards erythroblast maturation as indicated by cytospin preparations. Erythroid blasts are predominant at early stages of phase II and mature into orthochromatic normoblasts within two weeks. The pattern of maturation was similar to the one described by Wojda *et al.* (2002) despite the different protocol used.

The lower percentage of benzidine-positive cells can be attributed to the contamination of cultures with non-haematopoietic cells such as lymphocytes and monocytes. The presence of such cells can be observed in the cytospin preparations. Pope *et al.* (2000) showed that the majority of lymphocytes undergo apoptosis in Phase II and that the small numbers observed in the cultures are expendable to *in vitro* erythropoiesis in this two-phase culture system. Nathan *et al.* (1978) further suggested that activated T lymphocytes and monocytes served as sources of SCF *in vitro*. In order to produce more homogeneous and pure cultures, a number of studies have used suspension cultures with $CD34^+$ progenitors as the starting material. However, $CD34^-$ peripheral blood mononuclear cells yield larger numbers of erythroblasts than CD34-selected progenitors

(van den Akker *et al.*, 2010). This can be attributed to the fact that $CD34^+$ selection misses a significant portion of peripheral blood mononuclear cells with erythroid potential as suggested by Sonoda (2008).

HPLC analysis have shown that the percentage of HbF increases in the early stages of Phase II, peaks on day 6-7, and then decreases as the cells move towards more mature stages of erythropoiesis. Similarly, Dalyot *et al.* (1992) showed that γ-globin mRNA levels peak on day 6 of the two phase protocol. The drop in HbF percentage along with the concurrent increase in the percentage of HbA recapitulates expression patterns in the foetal to adult haemoglobin switch during ontogeny. However, despite the fall in HbF percentage at the end of Phase II, the percentage of HbF in erythroid cultures is still higher than the percentage in the peripheral blood of the same donor, a finding that was also documented by previous studies (Constantoulakis *et al.*, 1990, Hall and Motulsky, 1968, Papayannopoulou *et al.*, 1977).

Many studies point towards the presence of serum as the main cause of inconsistency in the liquid cultures, suggesting it as a source of undefined biochemical factors which can be inhibitory or stimulatory to haematopoiesis in cultures (Lebkowski *et al.*, 1995). Constantoulakis *et al.* (1990) suggested that foetal calf serum contains activity that increases the relative synthesis of HbF in erythroid cultures. Therefore, we investigated the percentage of FBS used in Phase II with the aim of identifying optimal FCS concentration associated with low percentage of HbF in adult cultures. During the early stages of phase II (day 3 and 6), higher percentage of FBS (45%) compromised cell survival, a finding that was not observed with 15% and 30% FBS, while maintaining similar percentages of haemoglobin containing cells and HbF. During later stages of phase II (day12), cultures with 30% FBS showed no major change in cell survival and haemoglobin containing cells compared to cultures grown in 15% and 45% FBS but showed lower percentage of HbF compared to the 45 % FBS. As a result, 30% FBS was chosen as the most suitable concentration of FBS. We further investigated three different types of FBS on their effect to modulate the ratio of γ-globin production. We have shown that indeed the type and treatment of FBS can have an effect on HbF levels. In contrast to the characterised and certified FBS, the heat inactivated FBS reduced cell survival and haemoglobinization while increasing the HbF percentage. Cheung *et al.* (2007) demonstrated that consistent failure of their serum-free cultures supplemented with IL-3, IL-6, SCF and EPO, to reproduce the high BFU-E expansion obtained by

others, suggests that the composition of the added serum can significantly influence cell growth in such cultures.

The effect of variable concentrations of EPO on the levels of HbF in primary human erythroid cultures was also investigated. It can be observed that different amounts of erythropoietin within the range of 0.1-1.5U/ml, does not affect the levels of HbF and HbA, as reported previously by Friedman *et al.* (1985). It was suggested that EPO stimulated the terminal differentiation of progenitors rather than promote HbF synthesis (Clarke *et al.*, 1979).

To identify the optimal treatment parameters that will generate the highest response to HU, we investigated the time point and length of duration of HU treatment in primary human erythroid cultures. HU exhibited the highest HbF inducing activity when added on day 6 of Phase II when erythroblasts have reached the basophilic stage. Treatment with the HU for 6 days showed a significant increase in HbF levels with minimal effect on cell survival when compared to shorter periods of treatment (4 days). Thus treatment of primary human erythroid cultures on day 6 of Phase II for 6 days was chosen as the optimal protocol for treatment with an agent.

3.2. Screening of caffeine and analogues in GM979 cells as potential HbF inducers

3.2.1. Introduction

Most HbF inducers have been identified based on their effect on γ-globin gene expression by altering local promoter chromatin or by altering the kinetics of erythroid differentiation. However, over the past few years, a number of cell signalling pathways have been demonstrated to have a role in regulating HbF induction. Among the implicated pathways include cyclic cGMP, cAMP, NO, p38 MAPK, ROS and cytokine signalling (Mabaera *et al.*, 2008a). In 2008, Mabaera *et al.* (2008b) proposed a cell stress signalling model of foetal haemoglobin induction involving a potential mechanistic link between CREB activation by p38 MAPK and cAMP-activated protein kinase A.

Caffeine, the most popular xanthine, is now known to have a wide range of biological effects due to its ability to target a range of molecules. At low concentrations, the main effect of caffeine is in the central nervous system through the inhibition of adenosine receptors. At higher concentrations, blockage of $GABA_A$ receptors and inhibition of phosphodiesterase activity, lead to increased cellular cAMP levels and release of Ca^{2+} from intracellular ryanodine sensitive stores (Daly, 2007). Moreover, increase in intracellular Ca^{2+} by caffeine, activates CREB through the binding of Ca^{2+}/cAMP response element (CRE) in the promoter of CREB-dependent genes (Connolly and Kingsbury, 2010). Based on the above data, it was decided that caffeine and related analogues should be investigated as potential inducers of HbF production, and thus eleven xanthines were selected for screening.

3.2.2. Screening of eleven xanthines in GM979 cells

Eleven xanthines with different molecular targets were chosen for screening using the modified GM979 cell line (Table 12). The GM979 cell line is a modified mouse erythroleukaemic cell line incorporating the $\mu LCR\beta_{pr}R_{luc}{}^{A}\gamma_{pro}F_{luc}$ construct (Figure 6) which contains firefly luciferase gene expression under the control of the human γ- and the renilla luciferase gene expression under the β-globin promoter (Skarpidi *et al.*, 2000). Four of the xanthines tested, caffeine, paraxanthine, theophylline and theobromine, are found naturally. Paraxanthine is a human metabolite of caffeine,

theophylline is found in tea leaf infusions while theobromine is derived from cocoa beans. The remaining xanthines tested include three A_1-adenosine receptor selective antagonists (DPCPX, KW-3902, DPSPX), an A_{2A}-adenosine receptor selective antagonist (CSC), an A_{2B}-adenosine receptor selective antagonist (PSB1115), an A_3-adenosine selective antagonist (PSB-11) and a phosphodiesterase (PDE) inhibitor (Zaprinast).

Varying concentrations ranging from 0.01mM to 10mM were tested for each xanthine. The concentrations used for each xanthine were selected based on the active concentrations of each agent used in the literature. Major problems were encountered during identification of a suitable solvent for these agents. Most xanthines are only soluble in organic solvents such as dimethyl sulfoxide (DMSO) and absolute ethanol which are highly toxic to the cells. Due to the lack of alternatives, three solvents (DMSO, NaOH and ethanol) were used for solubilisation of the xathines but were used at lower non-toxic final concentrations. Table 12 shows the solvent used for each xathine.

The effect of each agent was investigated by measuring the firefly and renilla luciferase luminescence corresponding to the increase in γ- and β-globin promoter activity, respectively, in the presence and absence of the drug. Due to the lower luminescence of the renilla luciferase activity, the total promoter activity is calculated by the equation ($\gamma^F+2\beta^R$) to normalise for the difference in the activity of the two enzymes (Section 2.2.2.2) (Skarpidi *et al.*, 2000). An agent was considered to be effective if the ratio of γ-promoter activity relative to the total globin promoter activity was increased by >2 fold relative to the un-treated control. None of the agents showed higher induction than the positive control, proprionic acid, which showed an average increase of 10.9 at a concentration of 10mM (Table 13). As shown by the dose response curve for each agent (Figure 20), theophylline, paraxanthine, DPCPX , KW-3902, DPSPX, PSB115, and CSC reduced the expression of γ-globin promoter relative to the total globin promoter activity (<1 fold change) when compared to the un-treated control. Caffeine and theobromine showed an increase in the activation of γ-globin promoter compared to the un-treated control but the induction observed was less than the defined threshold of >2. Caffeine showed the highest induction (1.9-fold) at 10mM, a concentration that was very cytotoxic (3.9% cell survival). Treatment with PSB11 and zabrinast showed an

increase of more than 2-fold change in the ratio of γ-globin over the total globin promoter activity but the increase was accompanied by high cytotoxicity.

Table 12, Eleven xanthines were tested as potential HbF inducers. All agents have a different target and based on their structural differences, show varying solubility to solvents. The target of each agent and the solvent used for the preparation of working concentrations of each agent are shown in this table.

Agent	***Function***	***Solvent***
Caffeine	Natural xanthine	H_2O
Theophylline	Natural xanthine	0.1M NaOH
Theobromine	Natural xanthine	0.1M NaOH
Paraxanthine	Natural metabolite	H_2O
DPCPX (8-cyclopentyl-1,3-dipropylxanthine)	A_1-adenosine antagonist	DMSO
KW-3902	A_1-adenosine antagonist	100% ethanol
DPSPX (1,3-dipropyl-8-sulfopropyl xanthine)	A_1-adenosine antagonist	0.1M NaOH
CSC (8-3-chlorostyryl caffeine)	A_{2A}-adenosine antagonist	DMSO
PSB1115	A_{2B}-adenosine antagonist	H_2O
PSB11	A_3- adenosine antagonist	DMSO
Zaprinast	Phosphodiesterase inhibitor	DMSO

Table 13, Summary of the effect of the 11 xanthines in GM979 cell line. The table presents the concentration of each agent that gave the highest $\gamma^F/(\gamma^F+2\beta^R)$ change with minimum cytotoxicity (≥50% cell survival). The change in $\gamma^F/(\gamma^F+2\beta^R)$ is expressed as fold change relative to the un-treated control as measured by luciferase assay.

Agent	***Concentration (mM)***	***Fold change in $\gamma^F/(\gamma^F+2\beta^R)$ ratio relative to un-treated control***	***Cell survival (% relative to un-treated control)***
Caffeine	1	1.28	95.9
Theophylline	0.01	0.9	103.4
Theobromine	1	1.35	118.9
Paraxanthine	0.01	1.03	55
DPCPX	0.01	0.86	103.4
KW-3902	0.01	0.37	48.2
DPSPX	1	0.74	94.7
CSC	0.1	0.77	71.1
PSB1115	0.01	0.93	68.9
PSB11	0.01	0.97	69.1
Zaprinast	0.025	1.3	127.7
Proprionic acid	10	10.94	53

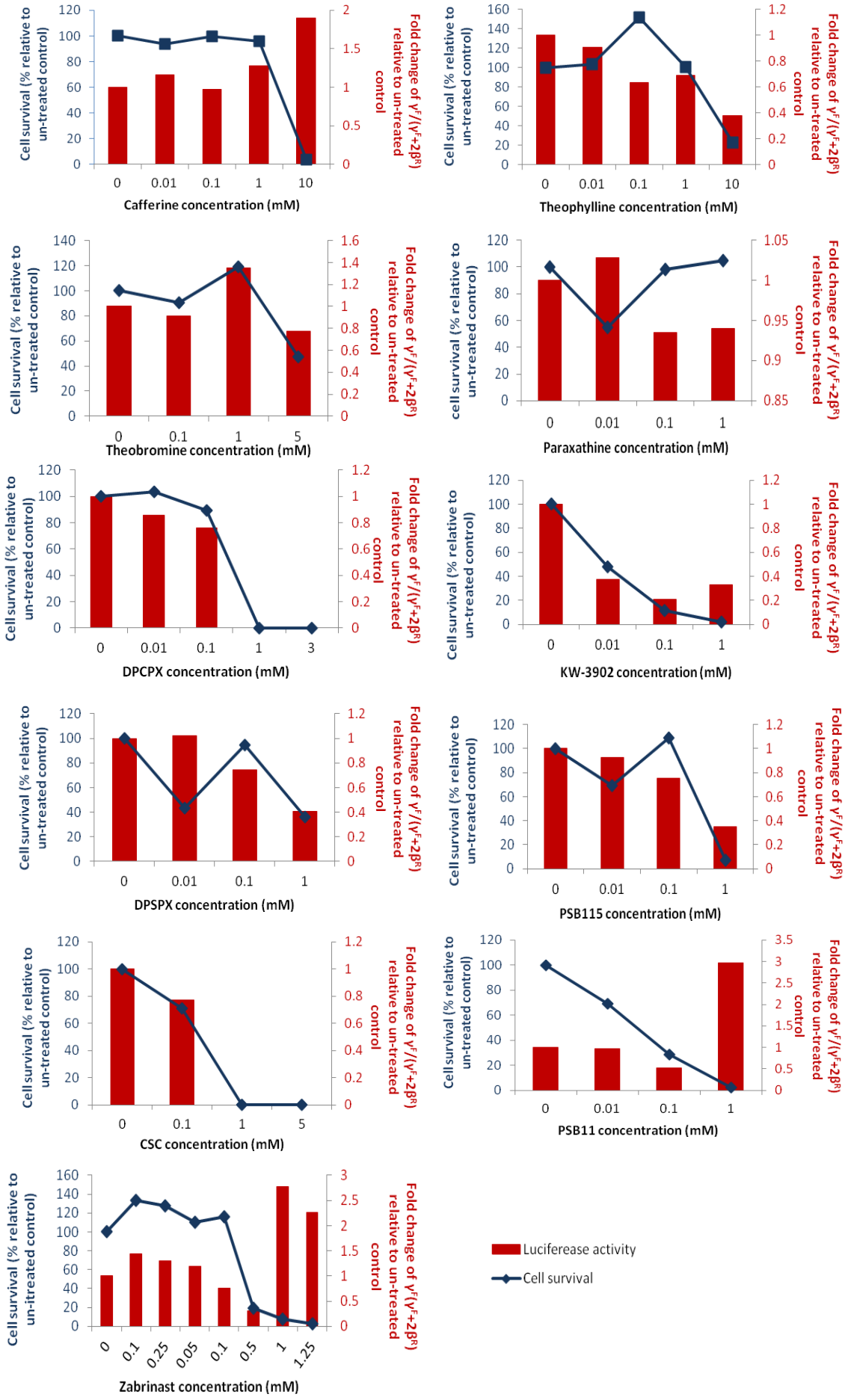

Cell survival (% relative to un-treated control)
Fold change of $\gamma^F/(\gamma^F+2\beta^R)$ relative to un-treated control
Caffeine concentration (mM)
Theophylline concentration (mM)
Theobromine concentration (mM)
Paraxathine concentration (mM)
DPCPX concentration (mM)
KW-3902 concentration (mM)
DPSPX concentration (mM)
PSB115 concentration (mM)
CSC concentration (mM)
PSB11 concentration (mM)
Zabrinast concentration (mM)
Luciferease activity
Cell survival

Figure 20, Dose response curves for the 11 xanthines tested in the GM979 murine cell line. The effect of each agent was tested at varying concentrations ranging between 0.01mM to 10mM and their effect on $\gamma^F/(\gamma^F+2\beta^R)$ ratio (bar graph) and cell survival (line graph) were investigated after 4 days of treatment with the agent by luciferase assay and trypan blue staining, respectively. The results represent the average of triplicates of single experiments for each agent.

Although the agents were generally toxic (<50% cell survival) at high concentrations, there was a peak in cell survival above the un-treated samples in most of the agents at concentrations 0.1-1mM that might suggest a proliferating effect of the agents. We concluded that the toxicity observed can be a result of the solvent alone or it can be attributed to the combination of the toxic effect of the agent and the solvent together.

3.2.3. Investigation of the effects of six caffeine analogues in K562 cells

Although the dual luciferase reporter GM979 cell line gives a good representation of the effect of each potential agent on HbF induction, measurement of luciferase activity by an enzymatic assay is limited by the significant degree of variability between experiments performed on different days (Skarpidi *et al.*, 2000). Furthermore, the particular model can only identify agents that act at the promoter region of the globin genes since the luciferase genes within the construct are under the influence of the γ-globin and β-globin promoters. In addition, only a small part of the μLCR locus is found within the construct that may not replicate full activity of β-globin LCR in the control of the globin gene expression.

We therefore decided to investigate the effect of the caffeine analogues in K562 cell line, an erythroleukaemic cell line that can spontaneously differentiate into recognisable progenitors of the erythrocyte lineage, therefore providing a more suitable environment for investigation of potential HbF inducers. Due to limited availability of the xanthines, only six of the caffeine analogous tested in the GM979 cell line could be tested in K562 cells.

Based on the results obtained from GM979 cells, the effect of the six xanthines was investigated in concentrations ranged from 0.01 to 1mM. The effect of each agent was defined by the increase in the percentage of haemoglobin (Hb) containing cells as

determined by benzidine stain along with the corresponding cell survival levels. Benzidine staining is the most widely used method for scoring erythroid differentiation. Haemoglobin containing cells will stain blue in the presence of benzidine stain and can be counted using a haemocytometer (Section 2.3.2.2). Therefore, an increase in the percentage of blue-stained cells represents an increase in haemoglobin production and thus erythroid differentiation.

As can be observed by the dose response curves (Figure 21), almost all of the xanthines tested in K562 cells show similar trends to the ones observed in GM979 cells. None of the natural xathines show any major increase in the percentage of Hb containing cells. Furthermore, the percentage of Hb containing cells appears to drop below the level observed in the un-treated control at high concentrations of the agents. DPCPX increased the percentage of Hb containing cells 4.7 fold above the levels observed in the un-treated control but this can be attributed to the high cytotoxicity observed, resulting in 28% cell survival at 0.01mM, a relatively low concentration.

Moreover, the increase observed was no more than that observed for the positive control (HU) which increased the percentage of Hb containing cells at an average of 4.9-fold relative to the un-treated control (Figure 21). DPSPX was the only xathine that showed an increase of 3.6-fold with moderate cytotoxicity (~60% cell survival) but again the effect observed was not better than the effect seen with the positive control. Interestingly, all of the natural xathines (caffeine, theobromine, paraxanthine and theophylline) showed an increase in cell survival at 0.1mM (theophilline and paraxanthine) or 1mM concentrations (caffeine and theobromine), a finding which was also observed in MEL cells suggesting a proliferative property of these agents at the particular concentrations.

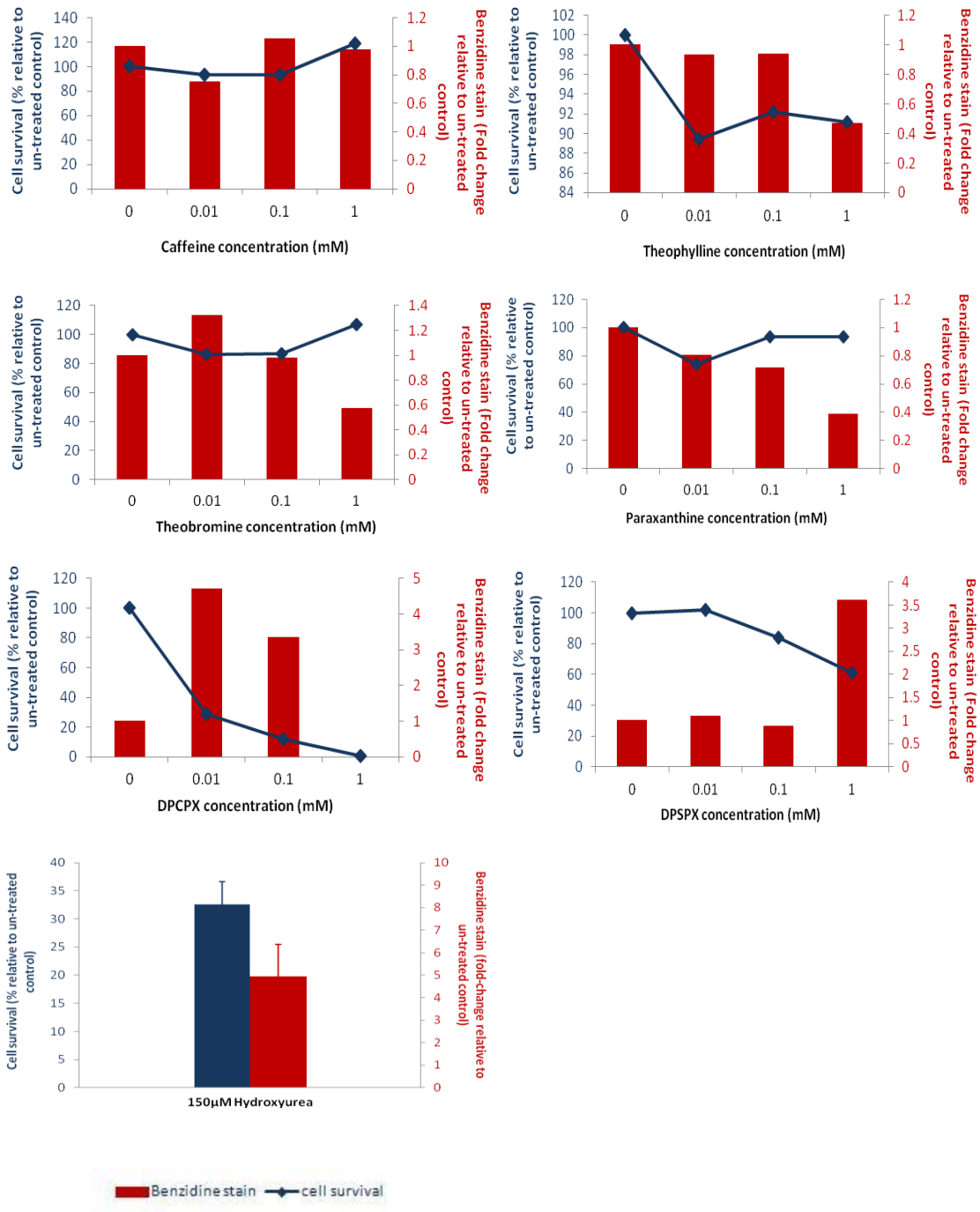


Figure 21, Dose response curves for six xanthine analogues in K562 cell line. The effect of each agent was represented as the fold change in the percentage of Hb containing cells relative to un-treated control as determined by benzidine staining (bar graph). Trypan blue staining was used to determine the cell survival as a percentage relative to the un-treated control (line graph). Each experiment had a positive control (150µM HU) and a negative control (un-treated control). The results represent the average of triplicate repeats of single experiments for each of the agents, and the average of three experiments for the positive control with error bars corresponding to standard deviation.

3.2.4. Discussion

Previous correlation of cAMP pathway to HbF induction led us to study the ability of caffeine and xathine analogues to induce HbF levels in GM979 cells containing a construct where luciferase gene expression was placed under the control of the β- and γ-globin gene promoters.

Caffeine, a natural xanthine, was initially isolated from coffee as a behaviour stimulant. It was not until the 1980s that its stimulant properties were fully understood. It is now known to be the most popular psychostimulant in the world with a wide range of molecular targets including adenosine receptors, where xanthines act as antagonists; phosphodiesterases (PDE), where xanthines act as inhibitors; calcium release ryanodine-sensitive channels in the sarcoplasmic and endoplasmic reticulum, where xanthines act to sensitise channels to the activation by calcium; and $GABA_A$ receptors, as antagonists at the benzodiazepine-positive modulatory site (Daly, 2007). At low doses, caffeine acts by inhibition of adenosine receptors in the central nervous system while at higher concentrations it blocks the $GABA_A$ receptors, inhibits phosphodiesterase activity leading to increased cellular cAMP levels and the release of Ca^{2+} from intracellular ryanodine sensitive stores stimulating Ca^{2+} signalling in a number of cell types (Ferre, 2008). Caffeine was recently demonstrated to stimulate CREB-dependent transcription in neurons mediated by the release of Ca^{2+} from ryanodine sensitive stores (Connolly and Kingsbury, 2010) and consequently increase the transcription of CREB target genes such as *Bdnf*. Moreover, caffeine was shown to have anti-tumour activity by affecting cell cycle progression and inducing apoptosis by activating the p38MAPK/c-Jun pathway and suppressing the ERK/c-Fos pathway (Liu and Chang, 2010).

Due to the poor solubility of the agents, a wide range of solvents was used for the preparation of the working solution for each agent. In the cases where DMSO and ethanol was used, working concentrations of the compounds were prepared in very low final concentrations of the solvents to minimise cytotoxicity to the cells. Apart from zabrinast and PSB11, all the xanthines tested either reduced the activity of γ-globin promoter over the total globin promoter activity or minimally increased the γ-globin promoter activity (<2-fold threshold). Zabrinast and PSB11 were the only two xathines that increased the γ-globin promoter activity above the 2-fold threshold but this increase was attributed to the cytotoxicity of the compounds. Furthermore, although most of the

xathines tested were shown to be cytotoxic, some of the agents, including KW-3902, DPCPX, PSB11 and CSC were very cytotoxic. This can be attributed to the increased number of carbons in the alkyl chain at R1and R3. This is in agreement with Rogozin *et al.* (2008) where they showed that xathine derivatives exhibited increased inhibition of neoplastic colony formation as the number of carbons increased at groups 1 or 3.

GM979 cells are characterised by a large variability due to the instability of the reporter gene expression and heterogeneity of the mixed culture system (Skarpidi *et al.*, 2000) and are limited due to the absence of many of the endogenous β-globin locus regulatory sequences from the reporter construct (Vadolas *et al.*, 2004). Therefore, in order to confirm the effect observed in the above model, the effect of six xanthines was also investigated in K562 cells, a human erythroleukaemic cell line. The response observed in K562 cells was very similar to the effect observed in GM979 cells for the xathines, showing very low levels of globin gene induction. The occasional moderate HbF induction observed was attributed to the cytotoxic effects of the compounds or their solvents and did not exceed the induction caused by the positive control (HU). Interestingly, despite most of the agents being cytotoxic, some stimulated an increase in cell survival above that seen for the un-treated control at concentrations of 0.1 and 1mM in both cell lines. Most studies have shown that caffeine promotes apoptosis and DNA damage-induced G2 arrest, but Tiwari *et al.* (2014) demonstrated that caffeine has a differential concentration-specific effect on cell cycle progression, checkpoint activation, cell viability and oxidative stress in human and murine pulmonary epithelial cells. Moreover, caffeine was shown to have a protective effect against oxidant–induced damage both *in vivo* and *in vitro* with antioxidant effect being prominent at low doses as shown in human skin fibroblasts. Therefore, one possible explanation for the increase in cell survival might be the intrinsic antioxidant activity of some of this xathines. Further investigation on this pattern of response would be interesting to define and understand the effect observed.

3.3. Screening of Resveratrol and its derivatives as potential HbF inducers

3.3.1. Introduction

Oxidative stress has been observed in various types of thalassaemia as well as in other hereditary and acquired haemolytic anaemias. This can be mainly attributed to the iron overload due to increased iron absorption in the gastrointestinal tract (Papanikolaou *et al.*, 2005), and multiple blood transfusions (Fibach and Rachmilewitz, 2010), as well as to increased intracellular denaturation of unbalanced haemoglobin subunits resulting in dissociation of haem from globin and iron from haem (Rund and Rachmilewitz, 2005; Fibach and Rachmilewitz, 2010). The excess iron levels eventually exceed the iron-binding capacity of transferrin, leading to circulation of labile non-transferrin bound iron species that undergo chemical reactions that generate ROS. ROS subsequently affect various cell components, particularly the cell membrane, damage vital organs including the heart, liver and endocrine system (Hershko *et al.*, 1998, Rachmilewitz *et al.*, 2005). In addition, endogenous antioxidant mechanisms are depleted due to the increased need to neutralise the oxidative stress (Kushner *et al.*, 2001). Oral administration of vitamin E in transfusion-independent beta thalassaemia patients was shown to decrease ROS production and increase glutathione reductase levels in red blood cells (Tesoriere *et al.*, 2001). Moreover, natural antioxidants were further studied for their potential use towards elimination of antioxidant stress. Curcumin, a natural herb used as food additive, was found to decrease iron induced lipid peroxidation *in vitro* (Grinberg *et al.*, 1996) while administration of fermented papaya preparation has shown decreased ROS generation, membrane lipid peroxidation and increased glutathione in β-thalassaemia/HbE disease patients (Fibach and Rachmilewitz, 2010).

Resveratrol is a natural phytoalexin found in a variety of human dietary products such as in the skin of red grapes, peanuts and red wines as well as in medicinal plants where it is produced in response to infection or other stresses. Resveratrol was originally isolated in 1940 from the root of *Veratrum grandiflorum*, a poisonous medicinal plant (Siemann and Creasy, 1992). However, it was not until the beginning of 1990 that research into resveratrol intensified due to evidence of the cardioprotective effects of red wine (Nakata *et al.*, 2012). Since the 1990s, evidence from several studies have shown resveratrol to have many biological activities including anti-inflammatory (Birrell *et al.*, 2005), anti-proliferative (Schneider *et al.*, 2000), chemopreventive effect

(Berge *et al.*, 2004, Jang *et al.*, 1997), antioxidant (Fremont *et al.*, 1999, Pandey and Rizvi, 2010) and lifespan enhancement activities (Howitz *et al.*, 2003, Valenzano *et al.*, 2006, Viswanathan *et al.*, 2005). Resveratrol also has the ability to limit or prevent progression of cerebral ischaemic injuries (Hung *et al.*, 2002, Ray *et al.*, 1999), cardiovascular injuries (Lekakis *et al.*, 2005, Wang *et al.*, 2002), cancer progression (Jang *et al.*, 1997, Kimura and Okuda, 2001, Tessitore *et al.*, 2000), arthritis (Elmali *et al.*, 2007, Tian *et al.*, 2013), diabetes (Su *et al.*, 2006), neurodegernerative disorders (Gupta *et al.*, 2002, Zhao *et al.*, 2013) and a number of other aging-associated and stress resistance disorders. The mechanisms by which resveratrol is able to exert such a wide range of effects is not fully understood but studies have led to the identification of a large number of direct targets for this compound including cyclooxygenase (COX) enzyme (Jang *et al.*, 1997, Subbaramaiah *et al.*, 1998), silent mating type information regulation 2 homolog 1 (SIRT1) (Xia *et al.*, 2013), Forkhead box protein O (FOXO) (Franco *et al.*, 2014, Roy *et al.*, 2011), haem oxygenase 1(HMOX1) (Quincozes-Santos *et al.*, 2013). Many of its biological actions have been attributed to its antioxidant properties.

Apart from its antioxidant activities, resveratrol was found to inhibit ribonucleotide reductase more efficiently than HU, the bench mark HbF inducer which is clinically used in patients with sickle cell disease (Rodrigue *et al.*, 2001). Fibach *et al.* (2012) were the first to show that resveratrol, in addition to its antioxidant activity, can also stimulate the expression of γ-globin genes and increase HbF. The above properties render the compound a promising agent for use in the pharmacological reactivation of foetal haemoglobin.

In the current section, we investigated the ability of resveratrol and nine new hydroxystilbenic derivatives of resveratrol, to increase HbF levels in K562 cell line and primary human erythroid progenitor cells, with the aim of identifing a novel agent that will combine potent HbF inducing activity with low cytotoxicity while maintaining the antioxidant activity of resveratrol.

3.3.2. Screening of resveratrol and 9 hydroxystilbenic derivatives in K562 cell line

Initially the effect of resveratrol was investigated *in vitro* using K562 cell line. Due to its low water solubility, the initial stock solution of resveratrol was prepared in 100% methanol. Subsequent working concentrations were prepared in 50% methanol in order to reduce the final percentage of methanol added to the cells. Based on the literature, varying concentrations of resveratrol were chosen for screening, ranging from 1-100μM final concentrations. The effect of each concentration was determined by benzidine staining after five days of treatment with the agent as described previously (Section 2.3). Results showed resveratrol to be a highly active HbF inducer resulting in an average of 5.7-fold increase in the percentage of Hb containing cells and 50% cell survival relative to the un-treated control when used at a concentration of 5μM. This was higher than the average effect observed with 150μM HU, the positive control which showed an average of 4.78-fold increase in the percentage of Hb positive cells at a concentration 30-fold higher than resveratrol (Table 14). However, resveratrol was also found to be slightly cytotoxic with cell survival reduced to 35% at 10μM concentration relative to the un-treated control (Figure 22). Although the cytotoxicity of resveratrol increased in a dose-dependent manner, the increase in the percentage of Hb containing cells did not show a linear relationship to increasing concentrations of the agent (Figure 22). Rather, resveratrol gradually increased the percentage of Hb containing cells, reaching the highest effect at 5μM followed by a reduction in the percentage of Hb containing cells between 10-15μM concentrations and a second peak at 20μM after which the effect plateaued. However, the effect observed at 20μM of resveratrol was compromised by the high cytotoxicity, corresponding to around 11% cell survival (Figure 22). Therefore, 5μM concentration was selected as the best concentration of resveratrol for its highest Hb inducing activity with a cell survival of 50%.

Since, the use of resveratrol might be limited due to this cytotoxicity, nine hydroxystilbenic derivatives of resveratrol were synthesised by our collaborators at the University of Modena and Reggio Emilia (Italy), with the aim of identifying an agent that retains the potent HbF inducing activity but with relatively lower cytotoxicity than the parent compound. All nine derivatives had the same backbone structure to the parent compound but had small substitutions on the othro, meta and para positions of the two aromatic rings (Figure 23). Initial stock solutions of each agent were prepared in 100% methanol to avoid any dissolution problems. Working solutions were again prepared in

50% methanol when possible, except when the compound was showing signs of precipitation in the presence of water.

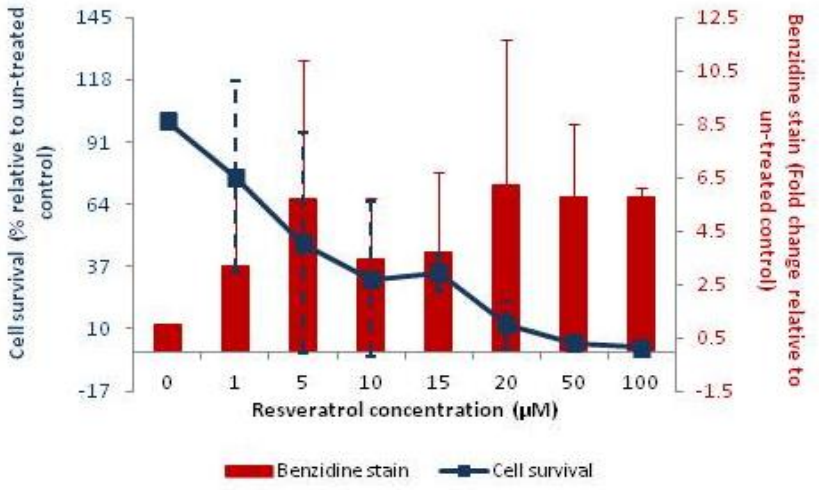


Figure 22, The effect of resveratrol in K562 cell line. After 5 days incubation with the agent, cell survival (line graph), presented as a percentage relative to the un-treated control and benzidine stain (bar graph), presented as a fold change in the percentage of Hb containing cells relative to the un-treated control. The results presented are the average of four experiments with error bars corresponding to the standard deviation.

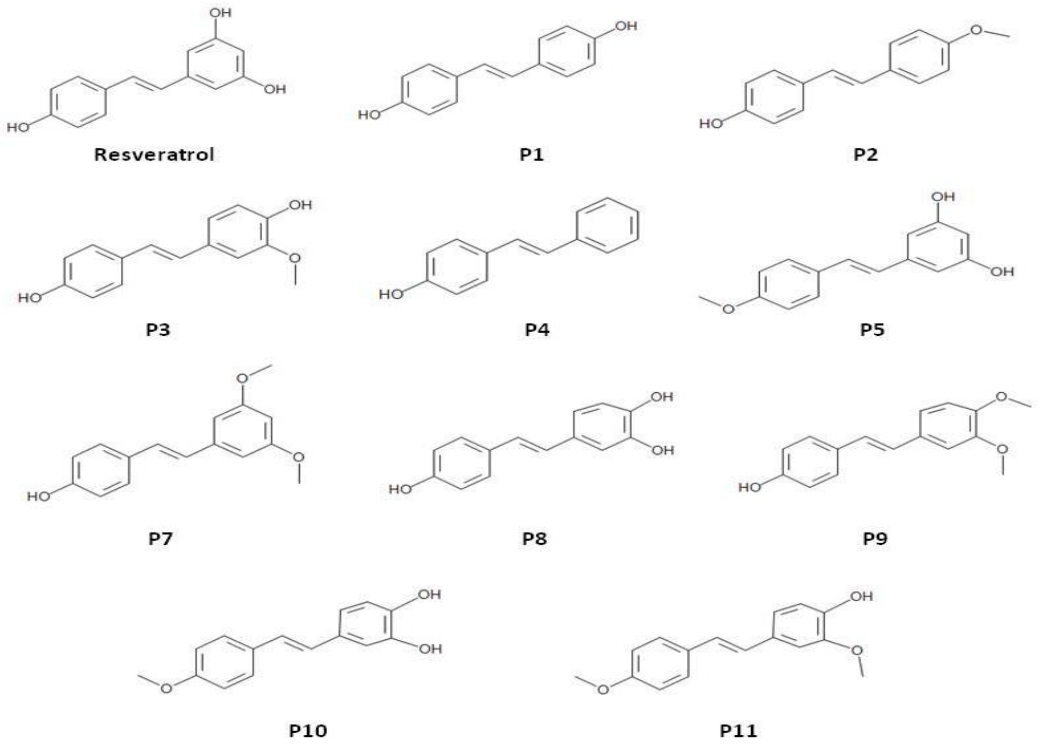


Figure 23, Structures of resveratrol and its nine hydroxystilbenic derivative analogues. In contrast to its derivatives, resveratrol, the parent compound is commercially available.

Initial screening of the nine derivatives was performed at different concentrations in K562 cell line after five days of treatment with the agents. Based on the results obtained by resveratrol and the limited compound stock concentration of each derivative, all derivatives were investigated at concentrations 1, 5, 10, 20, 40μM at the first round of screening. The best concentration of each agent was selected as the concentration that gave the highest increase in Hb induction with ≥50% cell survival. The dose response curves for each agent can be found in the Appendix III.

Derivatives P1, P2, P3, P4 and P9 showed similar cytotoxicity to resveratrol, reducing cell survival to approximately 50% at 5μM concentration (Table 14). P5, P7, P8 and P11 showed lower cytotoxicity than resveratrol, where cell survival approached to 50% at relatively higher concentrations of the agents (10-20μM) (Table 14). However, only P1, P4 and P11 exhibited Hb inducing activity comparable to the parent compound, with a 3.8, 2.9 and 3.3-fold increase in the percentage of Hb-containing cells relative to un-treated control, respectively (Table 14). Among the latter three compounds, P11 had the lowest cytotoxicity while maintaining comparable haemoglobin inducing activity to the rest of the compounds. This difference in cytotoxicity was apparent at concentrations of 5μM and above (Figure 24).

Table 14, Investigation of the haemoglobin inducing ability (Benzidine stain) and cytotoxicity (Trypan blue) of each resveratrol derivative in K562 cell line. The concentration of each agent resulting in approximately 50% cell survival and the corresponding effect in haemoglobin production is presented in this table. 150μM hydroxyurea was used as a positive control in all experiments. The values shown represent the average of three experiments.

Agent	***K562 cell line***		
	Concentration (μM)	***Cell survival (% relative to un-treated)***	***Benzidine stain (fold increase relative to un-treated control)***
Un-treated	-		1
HU	150	38.9	4.78
P1	5	43	3.8
P2	5	48.8	2.3
P3	5	44	2.8
P4	5	48	2.9
P5	20	65.5	2.56
P7	20	41.2	2.86
P8	20	48.9	1.9
P9	5	51.7	1.9
P11	10	52	3.3
Resveratrol	5	47.3	5.7

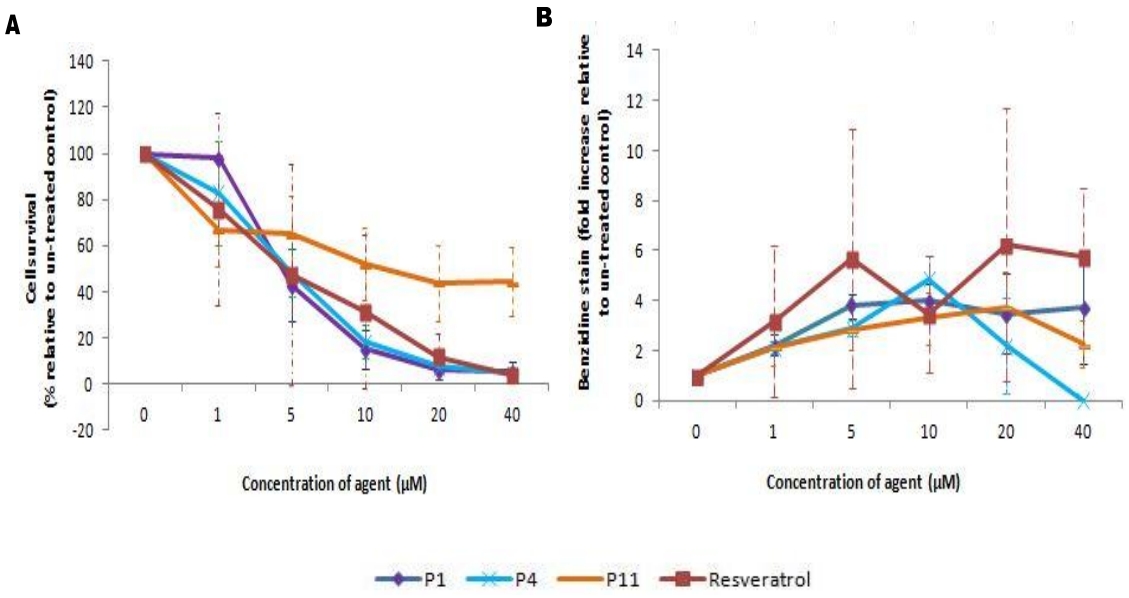


Figure 24, Investigation of haemoglobin inducing activity (A) and cytotoxicity (B) of compounds P1, P4 and P11 and resveratrol for a range of concentrations in K562 cell line. P1, P4 and P11 showed haemoglobin inducing levels similar to resveratrol, the parent compound, with P11 being less cytotoxic. The results are the average of three experiments with error bars corresponding to the standard deviation.

3.3.3. Screening of the three derivatives *ex vivo* in primary human erythroid progenitor cell cultures

The three derivatives (P1, P4 and P11) along with resveratrol were further studied in primary human erythroid cultures from healthy donors. The effect of each compound was investigated by cation exchange HPLC where the percentage of HbF was determined in the presence or absence of the agent. A wider range of concentration was set up for each agent in primary human erythroid cultures, in order to ensure the identification of the most effective concentration in the new system. Resveratrol was investigated at concentrations ranging between 10nM-100μM, P11 at concentrations 1nM-40μM, P4 at concentrations 10nM-40μM and P1 at concentrations 1-40μM.

HPLC analysis showed only a minimal change in the HbF percentage with all the agents (Figure 25). Resveratrol and P11 increased HbF levels by 6 and 18% at 1μM and 0.01μM respectively which were not statistically significant (p-value > 0.05, paired t-test) (Figure 25A & B). At higher concentrations of P11, 10 and 20μM, there was a 28 and 38% decrease in the HbF levels, respectively, when compared to the un-treated control (p-value < 0.05, paired t-test) (Figure 25B). P4 showed higher induction with

~40% increase in the HbF levels at 0.01μM (Figure 25C). Similarly, P1 showed a ~40% increase in HbF levels at 20μM (Figure 25D). The increase in the percentage of HbF observed in primary human erythroid cultures when treated with P4 and P1, however was not statistically significant (p-value > 0.05, paired t-test). Again this can be attributed to the large variability in the response observed in the relatively small sample size employed.

Although the HbF induction was minimal, resveratrol, derivative P4 and P11 were shown to be less toxic in primary human erythroid cultures than in K562 cells with no major reduction in cell survival for any of the concentrations of resveratrol tested, for concentrations below 20μM for P4 and for concentrations below 40μM for P11 (Figure 25). P1 showed higher cytotoxicity than resveratrol (Figure 25D) with cell survival dropping to less than 50% at concentrations of 5μM and above, and was therefore considered inappropriate as a therapeutic HbF inducer. Interestingly, induction with low concentrations of derivatives P11 was found to significantly increase cell numbers above those seen in the un-treated (negative) control (p-value<0.05, paired t-test) (Figure 25B). Although this was observed with resveratrol as well, the increse in cell numbers observed with both agents were not significantly elevated above those observed in cells treated with the vehicle alone (methanol) (p-value>0.05, paired t-test) (Figure 25). This was not however, true for P4, since cell numbers significantly (p-value<0.05, paired t-test) increased above those observed with methanol alone at 10nM of P4 suggesting a proliferating role at low concentrations of the agent.

150μM HU was used as positive control in all cultures which showed an average of 70-80% increase in the percentage of HbF. Therefore, although the three derivatives had shown a good response in K562 cell line, none of the agents showed an ability to increase HbF levels in primary human erythroid cultures. Moreover, the response observed with any of the three reagents was not comparable to the response observed with HU.

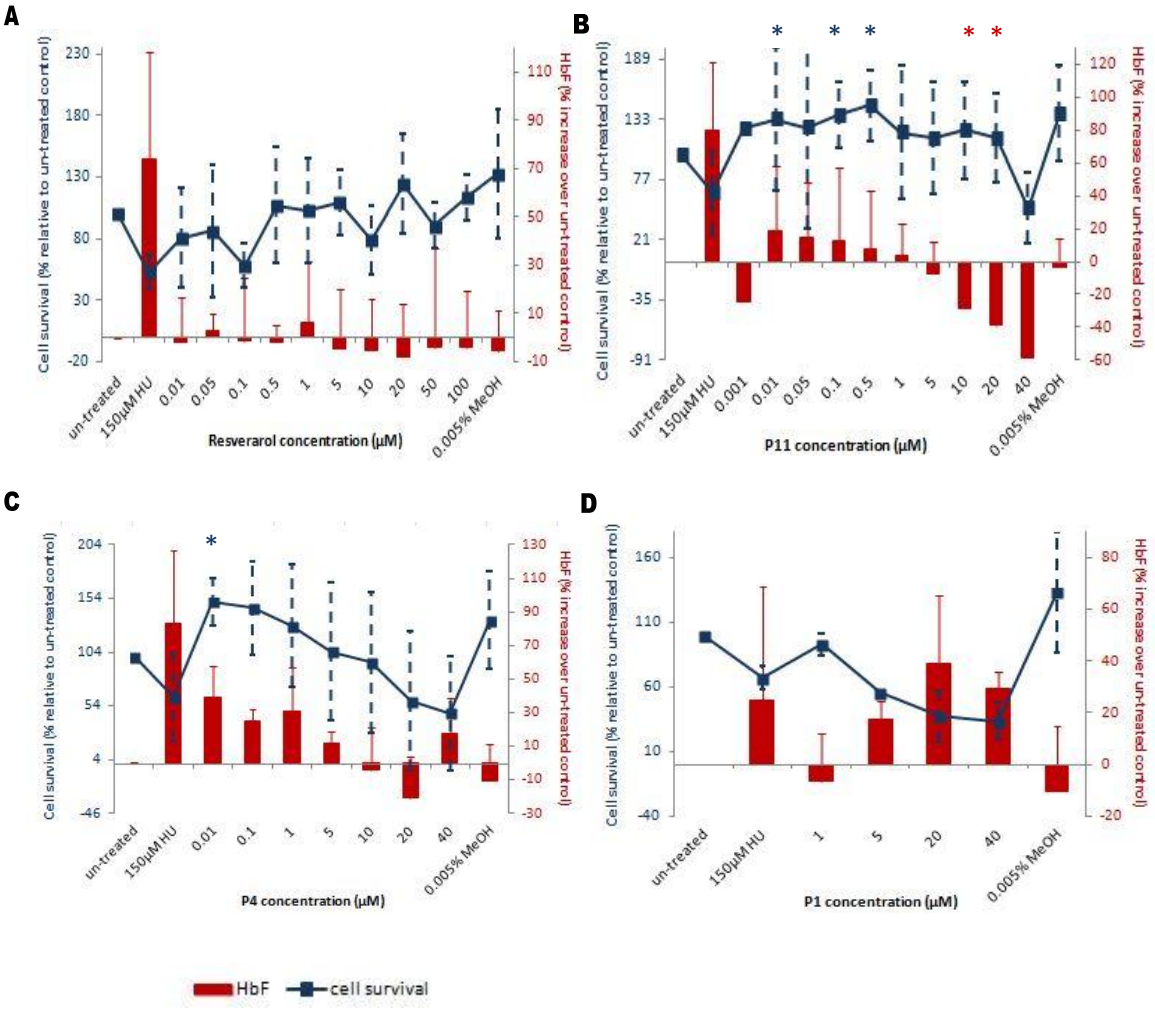


Figure 25, Treatment of primary human erythroid progenitor cells from healthy donors with resveratrol (A), P11 (B), P4 (C) and P1 (D). Cell survival (line graph), measured by trypan blue staining, and HbF levels (bar graph), measured by cation exchange HPLC, were investigated after 6 days treatment with the agent. 150μM HU was used as a positive control and un-treated cells were used a negative control. 0.005% methanol was the solvent for all agents. The results obtained are the average of 12-15 cultures for Resveratrol, P11 and P4 and the average of 2 experiments for P1 with error bars corresponding to the standard deviation. * corresponds to statistically significant changes in cell survival according to the paired t-test (p-value<0.05), * corresponds to statistically significant changes in HbF levels according to the paired t-test (p-value<0.05)

3.3.4. Effect of resveratrol on erythroid maturation

The lack of HbF inducing ability of resveratrol, prompted us to investigate its effect on erythroid maturation as a mechanism for its HbF inducing ability in primary human erythroid cultures. Flow cytometry analysis (CD71 proliferation and GPA differentiation markers) and cytospins for morphological changes were used as described in Sections 2.5 and 2.6. Staining of cytospin preparations from primary

human erythroid cultures treated with 1μM resveratrol showed an increase in the percentage of basophilic and polychromatic normoblasts compared to the un-treated cells (Figure 26A & B). Flow cytometry analysis of primary human erythroid cultures from healthy donors showed an average increase of 22.2% in the percentage of GPA^+ cells in the presence of resveratrol (Figure 26C) and a right shift in the mean intensity of the cells. In contrast, CD71 expression remained around the same levels with and without 1μM resveratrol (Figure 26C). These results suggest that resveratrol might be able to promote differentiation of primary human erythroid progenitor cells but without affecting their proliferation.

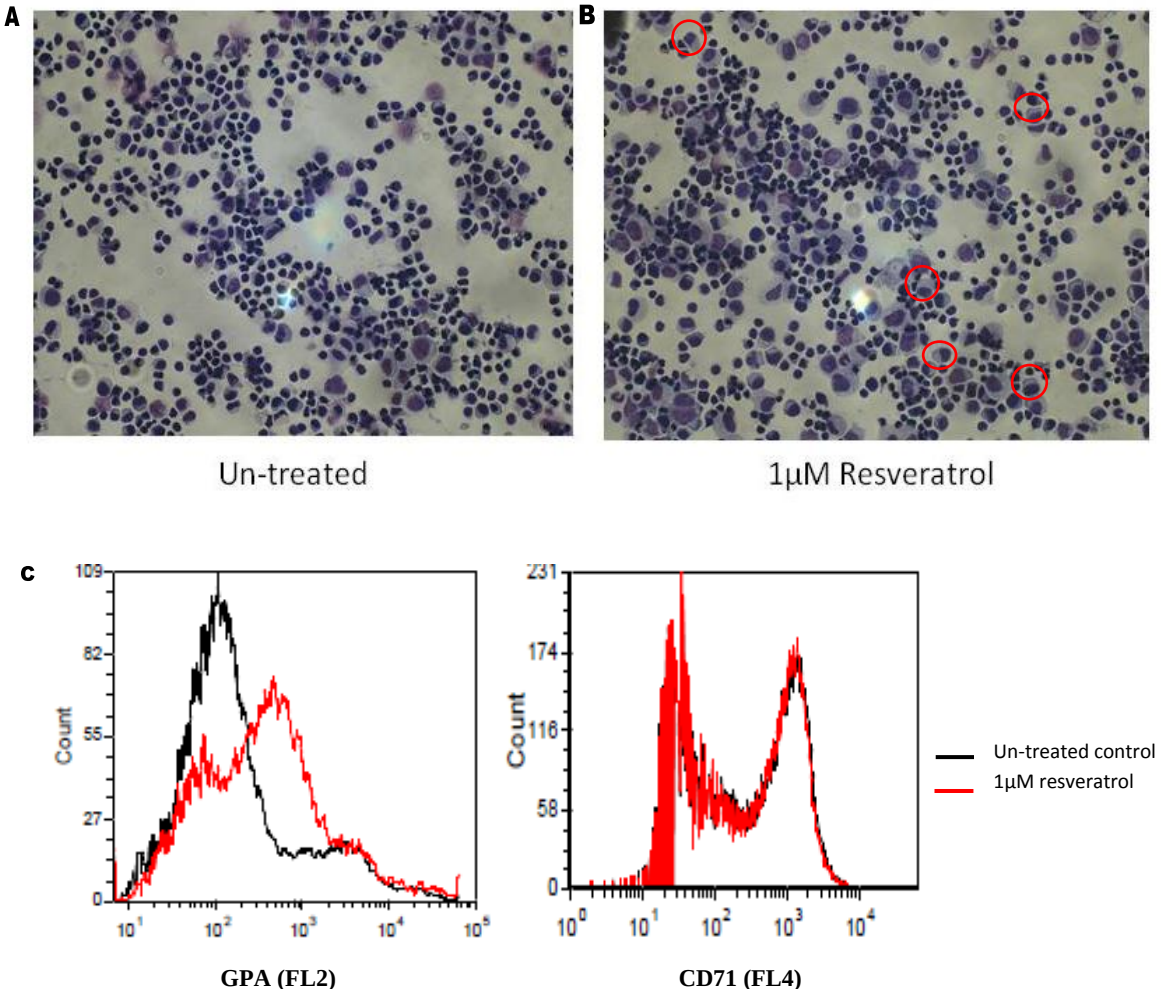


Figure 26, Investigation of the effect of resveratrol on erythroid differentiation in primary human erythroid cultures. Cytopsin preparation with eosin red and methylene blue staining of primary human erythroid cultures from healthy donors on day 12 of Phase II (A & B) show that resveratrol increases the percentage of basophilic and polychromatic normoblasts (B) compared to the un-treated cells (A). Flow cytometry shows the increase in the percentage of GPA positive cells in the presence of resveratrol but no major change in the CD71 proliferation marker (C). The red circles highlight the basophilic erythroblasts.

3.3.5. Combining the activities of resveratrol and decitabine in K562 cell line

Since none of the agents were found to be potent HbF-inducers in primary human erythroid cultures at non-toxic concentrations, the use of resveratrol in combination with another agent, was considered as a potential therapeutic approach to enhance the HbF-inducing activity. The antioxidant activity of resveratrol was combined with the strong HbF inducing activity of decitabine, a known HbF inducer, in K562 cell line. Screening of decitabine alone in this cell line showed a 5.3-fold increase in the percentage of Hb positive cells at 100nM concentration (50% cell survival) compared to the un-treated control (Figure 27). The combined use of decitabine with resveratrol increased Hb production significantly above the effect of resveratrol alone (p-value<0.05) at all concentrations. The increase in the percentage of Hb containing cells in the presence of the combination was also greater than the effect observed with decitabine alone but this increase was not consistently statistically significant (p-value>0.05). The strongest response was obtained when administering 100nM decitabine with 5μM resveratrol, resulting in a 6.5-fold increase in the percentage of haemoglobin containing cells, an increase that was significantly higher than the 1.83 observed with resveratrol alone. The increase was also higher than the 5.2-fold increase observed with decitabine alone, but this was not statistically significant (p-value>0.05). However, the combinatorial effect observed was significantly lower than the additive effect of both agents together (p-value<0.05, paired t-test) at all concentrations (Figure 27), suggesting competition between the two agents for common down-stream targets or saturation of common targets. Additionally, the cytotoxicity of the combinatorial regime was not significantly different from the additive toxicity of the two agents alone (p-value>0.05, paired t-test) and not always significantly greater than the cytotoxicity of each agent (p-value <0.05, paired t-test). Although the increased haemoglobin from the combined therapy was below the additive effect of the agents, the non-significant increase in cytotoxicity of the combination still renders the combinatorial approach as beneficial. Moreover, the small increase in Hb production exhibited by the combinatorial therapy might be attributed to the use of a very potent HbF inducing agent (decitabine) that might be overshadowing the effect of the less potent HbF inducers (resveratrol).

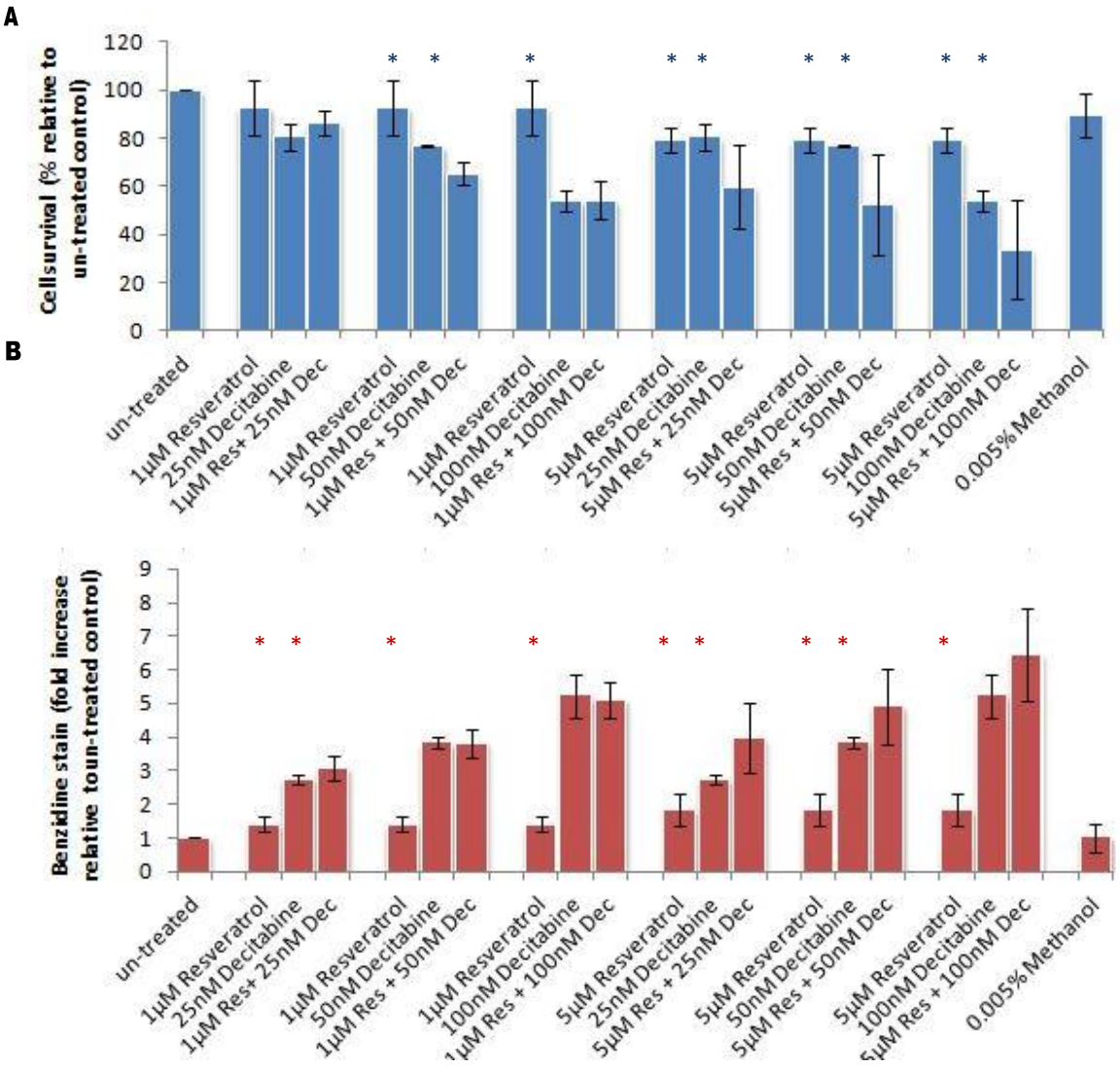


Figure 27, Dual treatment with resveratrol and decitabine in K562 cell line. Cell survival (A) and haemoglobin induction (B) were measured for each agent alone and in combination with the second agent. The results represent the average of three experiments with error bars corresponding to the standard deviation. * corresponds to statistically significant changes in cell survival according to the paired t-test (p-value<0.05), * corresponds to statistically significant changes in Hb induction according to the paired t-test (p-value<0.05).A significant increase in haemoglobin production (p-value< 0.05, paired t-test) was observed between the combined regime and concentrations of 1µM and 5µM resveratrol alone, but not significantly above all of the concentrations of decitabine alone. Cytotoxicity was not statistically increased above the additive effect of the agents and was not always significantly (p-value<0.05) increased above the toxicity of each agent alone.

3.3.6. Discussion

Studies have shown that resveratrol not only exhibit antioxidant activity, but can also stimulate the expression of the γ-globin genes (Fibach *et al.*, 2012). In the current study,

we have shown that increasing concentrations of resveratrol in the K562 cell line result in a dose dependent reduction in cell survival confirming its anti-proliferative effect, possibly through cell cycle arrest and induction of ER stress as shown by Liu *et al.* (2010). The anti-proliferative effect of resveratrol prompted the identification of derivatives which exhibit potent HbF-inducing properties with lower cytotoxicity. Out of the nine derivatives tested in K562 cells, only three (P1, P4 and P11) exhibited haemoglobin inducing activity comparable to the parent compound, with only one (P11) being less cytotoxic. Treatment of primary human erythroid cultures with these three agents had minimal effects on HbF levels when compared to the un-treated control. Although less cytotoxic than resveratrol, P11 increased the percentage of HbF only to a non-significant level when used at low concentrations while it caused a significant decrease in the percentage of HbF compared to the un-treated cells at concentrations above 10μM. In contrast, administration of P4 at 10nM increased the percentage of HbF levels by ~40% above that in the un-treated cells but lacked statistical significance due to the high variability in the response observed between cultures. Moreover, P4 also significantly increased cell numbers above those observed with vehicle-treated cultures at 10nM, a finding that was not observed for the other two derivatives. The three derivatives, although similar in structure, have important differences. One of the most easily observed structural differences which could explain some of the biological effects seen, is the lack of substituents on the aromatic ring in the case of P4 compared to the presence of ortho, para and meta substituents on resveratrol and the other derivatives. It would be interesting therefore to investigate whether this effect could be attributed to the structural differences. Despite its HbF-inducing activity, the high cytotoxicity of P1 renders it inappropriate as a therapeutic agent.

Although previous studies (Fibach *et al.*, 2012) have shown that resveratrol has an HbF inducing activity, our results show that none of the derivatives tested, including the parent compound, have a potent HbF inducing activity in primary human erythroid cultures from healthy donors. The lack of activity of resveratrol in primary human erythroid cultures, despite the high activity observed in K562 cell line, might be attributed to the different effects observed in the two systems. In K562 cells, resveratrol is shown to promote differentiation that might not necessarily correspond to an increase in the percentage of HbF in primary human erythroid cultures. In fact, the agent was shown to increase the percentage of basophilic and polychromatic normoblasts as well as the percentage of GPA^+ cells suggesting promotion of erythroid differentiation but

not proliferation as can be seen by the insignificant change in CD71 expression in the presence of the agent. This is in agreement with Franco *et al.* (2014) where they showed that resveratrol accelerates erythroid cell maturation through up-regulation of FOXO3a rather than increasing the expression of γ-globin gene expression. Furthermore, the results obtained in primary human erythroid progenitor cell cultures could be explained by the limited bioavailability of resveratrol in the current experimental model. Lancon *et al.* (2004) demonstrated that cellular uptake of resveratrol is reduced by two fold in medium containing 10% serum as compared to serum-free medium. Additionally, the authors showed that increasing concentrations of BSA reduced the uptake of resveratrol by two fold at a concentration of 3.5g/L albumin (Lancon *et al.*, 2004). The primary human erythroid cultures used for our screening experiments were grown in 30% serum and 11.3g/L BSA in Phase II. As a result, the lack of effect observed might also be attributed to the low uptake of the agent by the cells in the current experimental model, reducing the concentration of available drug. Our data therefore suggest that the effect of each agent should be confirmed in an alternative *ex vivo* experimental model.

Pharmacological attempts to find a suitable HbF inducer are limited by the insufficient HbF induction observed with current agents. We therefore investigated the combined administration of two agents as a therapeutic approach. Decitabine is a hypomethylating agent that is known to have a chemopreventive rather than carcinogenic effect while increasing HbF levels (DeSimone *et al.*, 2002). In contrast, resveratrol has an anti-oxidant activity, a crucial property in beta-thalassaemia, and can inhibit ribonucleotide reductase in a similar manner to hydroxyurea (Rodrigue *et al.*, 2001). Combining the two properties using a combination of resveratrol and decitabine in K562 cells increased HbF levels significantly above the effect of resveratrol alone. The combinatorial therapy increased Hb inducing activity above the effect exhibited by decitabine alone but only non-significantly. However, the Hb level observed with the combination was lower than the additive effect of the two agents suggesting competition between the two agents for common down-stream targets or saturation of common targets. Despite the minimal induction in haemoglobin production offered by the combinatorial regime, the combination should be investigated further in primary human erythroid cultures and thalassaemic mouse models to investigate the effect of the combinatorial therapy on HbF levels and clinical phenotype. The use of a combinatorial approach as a method for pharmacological reactivation of HbF is still interesting as can be seen by the increase in HbF levels in $CD34^+$ erythrocytes when treated with pomalidomide and HU (Moutouh-

de Parseval *et al.*, 2008). In the current study, the effect observed with the combined therapy might be overshadowed by the use of a strong inducer such as decitabine and thus alternative agents should be considered for this approach. Furthermore, studies regarding the HbF inducing activity of resveratrol are still controversial and thus further investigation of resveratrol and its derivatives as a therapeutic approach of β-thalassaemia is indicated.

3.4. Screening of four pre-selected HbF inducers in K562 cell line and primary human erythroid progenitor cells

3.4.1. Introduction

Attempts to identify novel HbF inducers are generally limited by our poor understanding of the mechanisms of action of these agents. One approach to overcome this limitation would be to better define the molecular mechanisms of already identified potent HbF inducers to allow the design of new therapeutic HbF inducers in a mechanism-based approach. Four promising HbF-inducing agents were thus selected based on the bibliography and preliminary studies with the aim of identifying the most active agent for delineation of its molecular mechanism of action. The four agents selected were: (1) Lenalidomide, member of a class of immunomodulators used as anticancer agents, (2) Angelicin, a DNA binding compound structurally related to psoralens, (3) 5-aza-2'-deoxycytidine (decitabine), an antimetabolite nucleoside analogue and (4) Mithramycin, another DNA-binding agent.

Several *in vitro* experimental systems have been utilised for screening of potential HbF inducers including immortalised cell lines, somatic cell hybrids, cultures of bone marrow- or peripheral blood-derived progenitors and thalassaemic mice models (Fibach, 1998). Rutherford *et al.* (1981), was the first to suggest the use of the human cell line K562 as a model for studying haemoglobin switching. K562 cell line has the ability to provide a reproducible, uniform, large population of cells that undergo stable and synchronised pattern of differentiation in the presence of chemical inducers such as haem (Rutherford *et al.,* 1981). It is this innate ability, the immortality and accessibility that renders the cell line an important model for the study of HbF inducers. K562 cell line was used for initial screening of the four pre-defined agents with the aim of confirming their HbF inducing potential and then selecting the agent with the highest activity.

3.4.2. Screening of the four pre-selected agents in K562 cell line

Based on the literature and previous experiments, a range of concentrations were selected for the investigation of each agent, to identify the concentration at which each agent will have maximun inducing activity with minimum cytotoxicity. Dose-response curves for each agent were prepared where the effect of each agent was determined as

an increase in the percentage of Hb containing cells by benzidine staining as described previously (Section 2.3).

Stock solutions of decitabine and mithramycin were prepared in H_2O, angelicin in ethanol and lenalidomide in DMSO. Working concentrations were prepared in such a way as to ensure a final concentration of 2-4% of ethanol and DMSO in the cells in order to eliminate any biases in the response due to the presence of the solvents.

The dose response curves (Figure 28) obtained in K562 cells showed that mithramycin and decitabine are the two agents with the highest Hb inducing activity, increasing the percentage of Hb containing cells by 9.91 and 8.66-fold above the un-treated negative control, respectively (Table 15). In addition, both mithramycin and decitabine showed a higher inducing activity than the positive control, HU, which increased the percentage of Hb containing cells by a 4.37-fold at 150μM (Table 15). Mithramycin showed the highest levels of induction at 10nM with a 24% cell survival, and decitabine at 400nM with a 15.7% cell survival (Figure 28). However, those concentrations are considered cytotoxic since they reduced cell survival below 50%. As a compromise, 5nM and 50nM were selected as the most efficient concentrations for mithramycin and decitabine, respectively (Figure 28), at which the HbF inducing ability is at highest with cell survival at no more than 50%. Angelicin and lenalidomide showed an average of 2.12 and 2.65-fold increase in the percentage of Hb containing cells, respectively, over the negative un-treated control, an effect which is lower than the Hb inducing ability of the positive control, HU (Table 15). Lenalidomide and angelicin were found to be active at higher concentrations (μM) than mithramycin and decitabine. Lenalidomide showed the highest inducing activity (7.3-fold increase over the un-treated control) at 300μM concentration, but cytotoxicity was unacceptable at that concentration dropping cell survival to ~3%. Angelicin showed the highest inducing activity (2.12-fold increase) at 300μM concetration with a 55.4% cell survival.

The results showed that mithramycin and decitabine had higher Hb inducing activities compared to angelicin and lenalidomide at a thousand–fold lower concentration rendering them the most effective agents.

Table 15, Screening of the four pre-selected agents in K562 cell line. The most efficient concentration for each agent was defined as the concentration that resulted in the highest Hb induction (benzidine stain) while maintaining a cell survival of 50% or over.

Agent	***Concentration (μM)***	***Cell survival (% relative to un-treated control)***	***Benzidine stain (Fold change relative to un-treated control)***
Hydroxyurea	150	46.13	4.37
Decitabine	0.05	46.1	8.66
Angelicin	300	55.4	2.12
Mithramycin	0.005	80.9	9.91
Lenalidomide	50	73.26	2.65

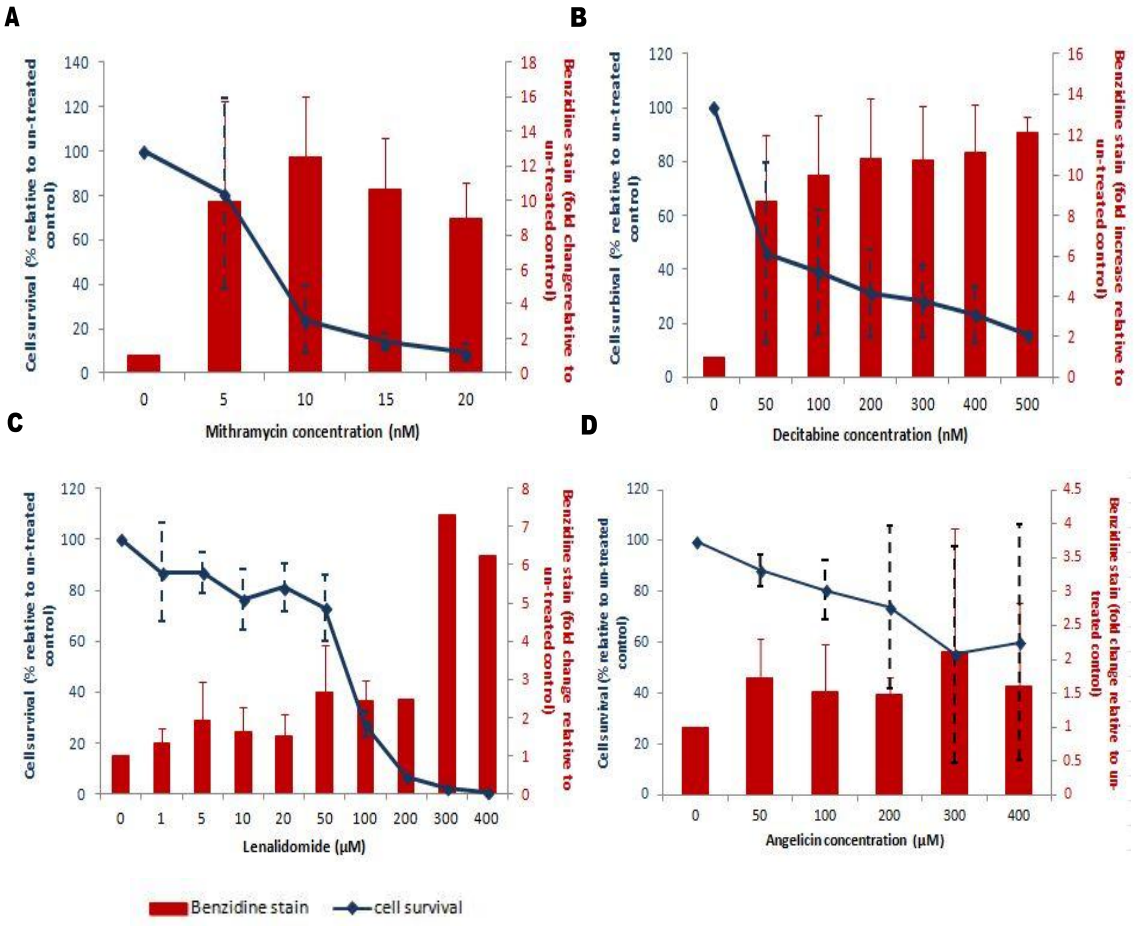


Figure 28, Investigation of the effect of the four pre-selected agents; (A) mithramycin, (B) decitabine, (C) lenalidomide and (D) angelicin in K562 cell line. Cell survival (line graph) and haemoglobin inducing activity (bar graph) were investigated after five days of treatment with each agent. Cell survival is presented as a percentage of the living cells in the presence of the inducer compared to cell numbers in the un-treated control while the haemoglobin inducing activity is presented as a fold increase in the percentage of haemoglobin containing cells relative to the un-treated negative control. The results are the average of 3-6 experiments, with error bars corresponding to the standard deviation.

3.4.3. Screening of the four pre-selected HbF inducers in primary human erythroid progenitor cells

Although K562 cells are the most highly used model for screening of potential agents, Rutherford *et al.* (1981) showed that the K562 cell line does not recapitulate all aspects of erythropoiesis. The cell line lack adult haemoglobin production despite the presence of an intact β-globin gene within the cells, suggesting that the cell line originated from very immature progenitors (Rutherford *et al.*, 1981). Cioe *et al.* (1981) suggested that different lines of K562 cells respond differently to inducers, probably due to their variable karyotype. In addition, Testa *et al.* (1982) demonstrated a large heterogeneity in the type of haemoglobin produced by different clones of K562 cell line. The presence of an intact β-globin gene in the absence of β-globin chain synthesis in K562 cells led Rutherford *et al.* (1981) to suggest that the predominant synthesis of foetal and embryonic globins in K562 cells might be a consequence of epigenetic control analogous to that present in the normal embryonic erythroblasts. The epigenetic changes might result in a block of the embryonic to foetal haemoglobin switch and expression of mostly embryonic globins with minimal expression of foetal and absent adult globins even in the presence of haemoglobin inducers. Moreover, agents identified in this model as effective haemoglobin inducers, are not always effective in primary human erythroid cultures, thus raising questions about the reliability of the model.

Due to the presence of these limitations of K562 cell line, the effect of the four pre-selected HbF inducers was also investigated in primary human erythroid cultures, to determine whether the inducing activity of the agents in K562 cells corresponds to an increase in HbF production in primary human erythroid cultures.

The effect of the four agents was determined as a percentage increase in HbF levels relative to the un-treated control, measured by cation exchange chromatography. For each agent, dose-response curves were prepared in primary human erythroid cultures using the same concentration range used in K562 cells. Screening of the four agents in primary human erythroid cultures from healthy donors (Figure 29) confirmed the results obtained in K562 cell line. Decitabine and mithramycin showed the highest HbF inducing activity raising the percentage of HbF by 95 and 62 % above the HbF levels of the un-treated negative control at 400nM and 20nM, respectively (Figure 29A & B). The increase in the percentage of HbF observed with decitabine increased with increasing concentration of the agent. Although, cell survival decreased with increasing

concentrations of decitabine, the agent was shown to be the least cytotoxic among the four agents in primary human erythroid cultures; cell survival was reduced to only 71% at 400nM concentration. In contrast, despite using 10-fold lower concentrations, mithramycin was relatively more cytoxic reaching a 50% cell survival at a concentration of 15nM. In contrast to decitabine and mithramycin, increasing concentrations of lenalidomide decreased HbF induction. The highest increase in the percentage of HbF was seen (46%) at 5µM of lenalidomide (Figure 29C). The highest HbF inducing activity of angelicin was observed at 400µM where the agent increased the percentage of HbF relative to the un-treated control by 52% but reduced the cell survival to 44% (Figure 29D).

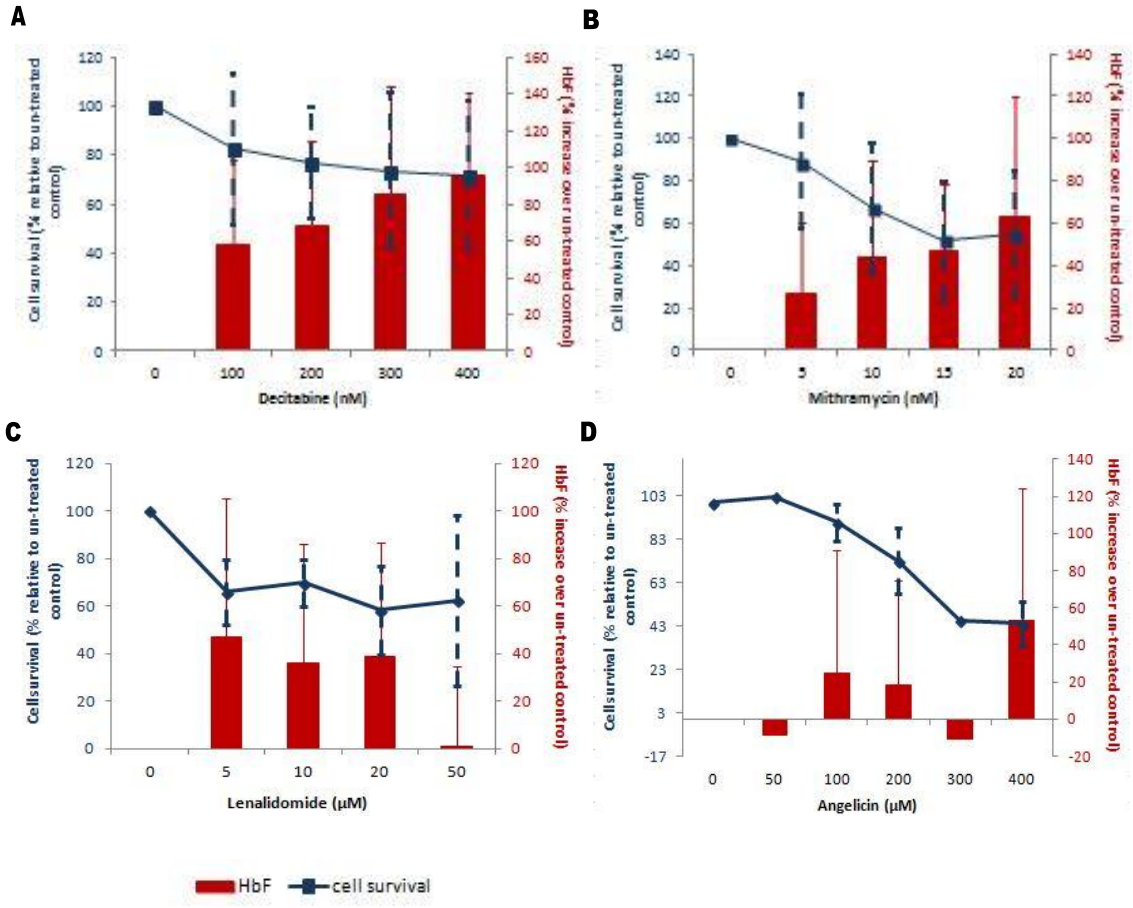


Figure 29, Dose response curves for decitabine (A), mithramycin (B), lenalidomide (C) and angelicin (D) in primary human erythroid progenitor cells from healthy donors. The effect of each agent on the percentage of HbF (bar graph) was determined by cation exchange HPLC and on cell survival (line graph) by trypan blue staining. Decitabine and mithramycin have the highest HbF inducing activity with decitabine exhibiting lowest cytotoxicity. The results are the average of 6-12 experiments with error bars corresponding to the standard deviation.

The effect of the agents were also investigated in primary human erythroid progenitor cells from thalassaemic patients and dose-response curves were prepared for decitabine and mithramycin (Figure 30). Decitabine was once more shown to have higher HbF-inducing activity compared to mithramycin while showing lower cytotoxicity. The highest inducing activity for decitabine was observed at a concentration of 300nM where the percentage of HbF was increased by 68% and cell survival reduced to 81% relative to the un-treated control (Figure 30A). Mithramycin had the highest inducing activity at 20nM concentration, but at this concentration cytotoxicity was unacceptably high with cell survival dropping to 33.3% (Figure 30B).

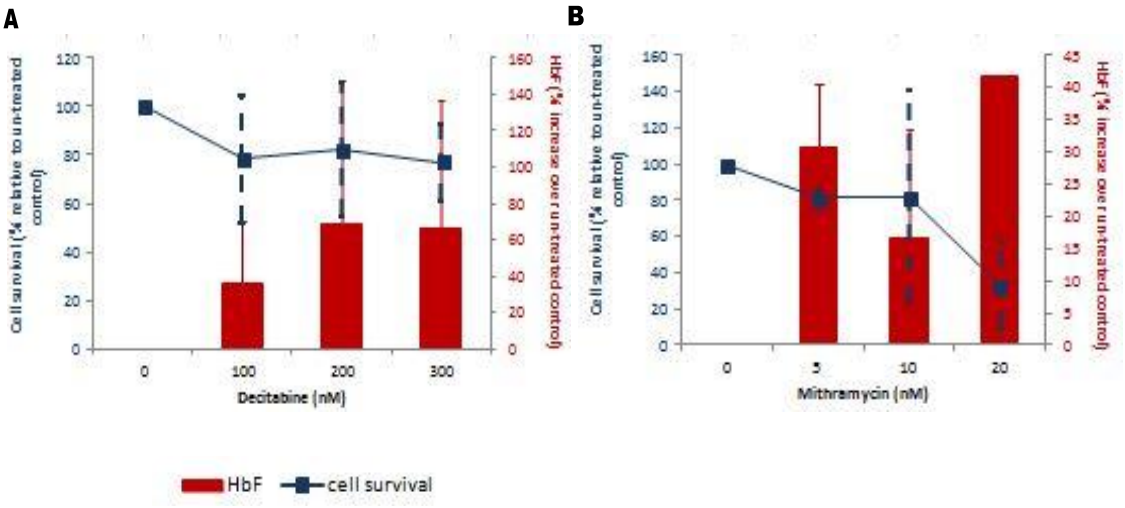


Figure 30, Dose response curves of decitabine (A) and mithramycin (B) in primary human erythroid cultures from thalassaemic donors. The effect of each agent on the percentage of HbF (bar graph) was determined by cation exchange HPLC and on cell survival (line graph) by trypan blue staining. The results are the average of 3 experiments with error bars corresponding to the standard deviation.

Decitabine was therefore selected as the agent with the highest inducing activity and lowest cytotoxicity profile in primary human erythroid progenitor cells from both healthy and thalassaemic donors at the optimal concentration of 300nM.

3.4.4. Discussion

Initial screening of the four pre-selected agents in K562 cells led to the identification of mithramycin and decitabine as the two agents with the highest HbF inducing activity, resulting in approximately 9-fold increase in the percentage of Hb-containing cells

relative to the un-treated control. The effect observed was higher than that observed with HU (at 1000-fold lower concentration than HU). Decitabine was shown to increase Hb-inducing activity with increasing agent concentration, while compromising cell survival at high concentrations. This is in agreement with Pinto *et al.*(1984) who showed dose-dependent haemoglobinization and differentiation of K562 cells with increasing concentrations of decitabine. Mithramycin, a DNA binding drug that targets G+C rich nucleotide sequences, was suggested as a promising HbF inducer. Mithramycin induced erythroid differentiation of K562 cells in a dose-dependent manner, peaking at 10nM, but inhibited cell proliferation by >50% at that concentration, a finding that was also observed by Bianchi *et al.* (1999).

In contrast, angelicin and lenalidomide exhibited lower HbF inducing activities compared to HU at much higher concentrations than mithramycin and decitabine. Angelicin was active at 300μM with an average cell survival of ~60%. Similarly, Lampronti *et al.* (2003) also demonstrated that the highest inducing activity of angelicin was at 200-400μM, but still lower than that of mithramycin. Lenalidomide increased erythroid differentiation by 2.65-fold at 50μM but was not cytotoxic until 100μM. Despite the low cytotoxicity, lenalidomide's Hb inducing ability was below that observed with mithramycin and decitabine. Based on these results, mithramycin and decitabine were selected as the two agents with the highest inducing activity at lower concentrations than any of the other selected agents in K562 cell line.

Screening of the agents in primary human erythroid cultures confirmed the effect observed in K562 cells with decitabine and mithramycin showing the highest HbF inducing activity. In contrast to the literature, mithramycin was shown to be highly cytotoxic at 10-20nM. Survival of primary human erythroid cultures was at 50% at only 15nM concentration. The increase in the percentage of HbF observed at 15nM of mithramycin was similar to the effect observed by Fibach *et al.* (2003) when primary human erythroid cultures were treated with the agent on day 4 of Phase II. However, the authors in the latter study showed higher induction at higher concentrations of mithramycin, concentrations that were found to be very cytotoxic in the current study.

Lenalidomide and angelicin were found to have moderate HbF-inducing activity in primary human erythroid cultures, causing an increase of 46% and 52%, respectively, in the HbF level over the un-treated negative control. In contrast to Moutouh-de Parseval *et al.* (2008), we showed that cell survival decreased with increasing concentrations of

lenalidomide. We observed the highest inducing activity of lenalidomide at 5μM with increasing concentrations of the agent resulting in decreasing HbF levels. Moutouh-de Parseval *et al.* (2008) did not investigate the effect of the agent at concentrations higher than 10μM, therefore failing to observe the dose-dependent decrease in HbF levels.

In agreement with the literature (Lampronti *et al.*, 2003, Lampronti *et al.*, 2009) angelicin had a lower HbF inducing activity than mithramycin but higher than hydroxyurea in primary human erythroid cultures from healthy donors. However, in contrast to Lampronti *et al.* (2003), the effect observed did not reach a 10-fold increase relative to un-treated control.

Based on the results obtained in the current experiments, decitabine had the highest HbF inducing activity among the four agents in primary human erythroid cultures from both healthy and thalassaemic donors. Decitabine had been previously shown to stimulate HbF production in patients with sickle cell disease that did not respond to HU (Koshy *et al.*, 2000, Saunthararajah *et al.*, 2003). Olivieri *et al.* (2011) demonstrated that decitabine can also increase HbF levels in β-thalassaemia intermedia patients without being cytotoxic. The only notable side effect of decitabine in clinical trial was the increase in platelet count that did not correlate with any clinical symptoms (Olivieri *et al.*, 2011). Therefore, based on the results obtained from both K562 cells and primary human erythroid progenitor cells, decitabine was selected for further study and delineation of its molecular mechanisms of action in primary human erythroid cultures.

3.5. Characterisation of primary human erythroid cultures in the absence and presence of decitabine

On selection of decitabine as the most efficient HbF inducer, we moved on to characterise its effect in primary human erythroid progenitor cells from healthy and transfusion-dependent thalassaemic donors. Following the previously defined protocol (Section 3.1), primary human erythroid cultures were treated with the agent on day 6 of phase II and incubated with the agent for 6 days for maximum HbF induction. The effect of the agent on globin gene expression was initially investigated using HPLC analysis, qRT-PCR and western blot analysis, to define the changes in HbF levels and γ-globin gene expression at the protein and mRNA levels, respectively.

Despite the large amount of experimental data on the association of DNA methyltransferase inhibitors and HbF induction, the exact mechanism underlying the increased expression of HbF (and gamma globin genes) by such agents is still debatable. In order to correlate the effect of decitabine on globin gene expression with its hypomethylating function, we investigated the changes in DNA and histone methylation patterns caused by decitabine in primary human erythroid progenitor cells from healthy donors. In addition, the expression of ten erythroid-related genes known to have a role in γ-globin gene reactivation (*BCL11A*, *KLF1*, *GATA1*, *SP1*, *MYB*, *NRF2*, *SOX6*, *MBD2*, *HMOX1* and *CREB*) was investigated in primary human erythroid progenitor cells from healthy and transfusion-dependent thalassaemic donors treated with decitabine as an approach to derive some insight on the mechanism of action of decitabine.

3.5.1. Investigation of the effect of decitabine on globin gene expression in primary human erythroid cultures

The effect of decitabine on γ-globin gene expression was studied in primary human erythroid cultures from twelve healthy donors and twelve IVS1-110/IVS1-110 transfusion-dependent thalassaemia patients with the aim to select the cultures with the highest increase in the percentage of HbF for proteomic analysis. HPLC analysis (Table 16) showed that decitabine increased the percentage of HbF by an average of 17.34 and 62.68% after 3 and 6 days of treatment, respectively, in cultures from healthy donors and by an average of 33.8 and 43.9%, after 3 and 6 days of treatment, respectively, in

thalassaemic cultures. The increase in HbF percentage ranged between 24.6-114.2% in healthy cultures and between no response to 149% in thalassaemic cultures (Table 16), suggesting different susceptibility of each individual to the agent. Furthermore, the response of some cultures to the agent increased with increasing incubation time while in others the response decreased with increasing length of treatment with the agent. This variability in the response was also prominent in thalassaemic cultures. The increase in both healthy and thalassaemic cultures was statistically significant (p-value<0.05) (Table 17) only after 6 days of treatment with decitabine. Although the HbF induction in thalassaemic cultures treated with the agent was at lower levels than those observed in cultures from healthy donors, the HbF levels obtained in the presence of decitabine (14-45%) might be sufficient and clinically beneficial.

In addition, decitabine increased the expression of γ-globin gene at the mRNA level both in healthy and thalassaemic patients (Figure 31). γ-globin expression increased by an average of 2.97 and 3.69-fold relative to the un-treated control in healthy and thalassaemic cultures, respectively, after 6 days of treatment. Similar to HbF, γ-globin mRNA levels increased with increasing length of treatment in both healthy and thalassaemic cultures. A concurrent increase in α-globin gene expression was also observed in healthy cultures following both 3 and 6 days of treatment with the agent. In constrast, in thalassaemic cultures, α-globin gene expression dropped to half after three days of treatment, while its expression increased to 1.5-fold following 6 days of treatment. There is a small decrease in β-globin expression in healthy cultures but no major change in β-globin gene expression in thalassaemic cultures following treatment with decitabine.

In concordance with HPLC results, western blot analysis of primary human erythroid cultures showed a general increase in HBG expression in both healthy and thalassaemic cultures (Figure 32). The increase was more pronounced in healthy cultures. The average increase of HBG expression in healthy cultures based on western blot analysis was 2.63-fold relative to the un-treated counterpart compared to an average of 1.33 in thalassaemic cultures (Figure 32) after 6 days of treatment.

Although there is a large variation in the response between individual cultures as demonstrated by all methods (HPLC, qRT-PCR and western blot analysis), the results broadly demonstrated a general increase in γ-globin expression both at the protein and mRNA level after 6 days of treatment with the agent. The lack of statistical significance

is probably due to the large variation in the response to the agent between individual cultures.

Table 16, The percentage increase of HbF in twelve primary human erythroid cultures from healthy donors and twelve primary human erythroid cultures from thalassaemic donors after 3 and 6 days of treatment with 300nM decitabine. The percentage increase in HbF was calculated relative to the HbF levels of the un-treated counterpart of the each culture.

Healthy donors		***HbF percentage***		***Increase in HbF (% relative to un-treated) 3 days***	***HbF percentage***		***Increase in HbF (% relative to un-treated) 6 days***
No	***ID***	***Un-treated***	***3 days of treatment***		***Un-treated***	***6 days of treatment***	
1	**974**				3.74	8.01	114.20
2	**965**				10.30	19.65	90.89
3	**242**	8.12	13.48	65.98	13.46	16.77	24.65
4	**245**	4.50	6.44	43.06	5.65	8.86	56.83
5	**888**	3.05	1.63	-46.57	3.18	5.81	83.12
6	**165**	4.51	5.09	12.87	5.31	7.00	31.93
7	**208**	12.35	12.62	2.16	11.99	19.36	61.49
8	**280**	3.48	4.62	32.57	5.07	9.10	79.51
9	**367**	1.94	2.12	9.49	2.53	4.22	66.79
10	**844**	8.37	6.06	-27.64	7.37	9.30	26.33
11	**885**	7.69	12.25	55.27	12.83	19.73	53.78
12	**882**	6.08	7.62	26.18			
	Average	***6.01***	***7.19***	***17.34***	***7.40***	***11.62***	***62.68***

Transfusion dependent Thalassaemic donors		***HbF percentage***		***Increase in HbF (% relative to un-treated) 3 days***	***HbF percentage***		***Increase in HbF (% relative to un-treated) 6 days***
No	***ID***	***Un-treated***	***3 days of treatment***		***Un-treated***	***6 days of treatment***	
1	**675214**	15.99	23.54	47.21	21.36	28.58	33.78
2	**724636**	22.58	30.48	34.99	23.48	36.67	56.13
3	**704965**	13.61	16.24	19.33	13.74	18.21	39.81
4	**730099**				15.39	20.62	34.00
5	**784676**				15.80	22.65	43.35
6	**838990**				9.87	15.46	56.64
7	**665190**				18.21	20.61	13.17
8	**739392**				17.61	26.99	53.29
9	**710148**				18.11	45.21	149.68
10	**754964**				12.82	14.10	9.99
11	**754676**				21.79	31.35	43.88
12	**822141**				26.03	24.20	-7.02
	Average	***17.39***	***23.42***	***33.84***	***17.85***	***25.39***	***43.89***

Table 17, The increase in HbF percentage in primary human erythroid cultures from healthy and thalassaemic donors as quantified by HPLC after 3 and 6 days of treatment with 300nM decitabine. The values are the average of twelve healthy cultures and twelve thalassaemic cultures and are presented as the average increase of HbF levels relative to the un-treated negative control ± standard deviation. The p-values demonstrate the presence (p-value<0.05) or absence (p-value>0.05) of statistical significance in HbF induction after 3 and 6 days of treatment according to the paired t-test.

Samples	***Increase in HbF (% relative to un-treated control)***		***p-value (Paired t-test +/- Dec)***	
	3 days	***6 days***	***3 days***	***6 days***
Healthy	17.34 ± 35.27	62.68 ± 28.66	0.177	0.00003
Thalassaemic	33.84 ± 13.97	43.89 ± 38.72	0.0554	0.0027

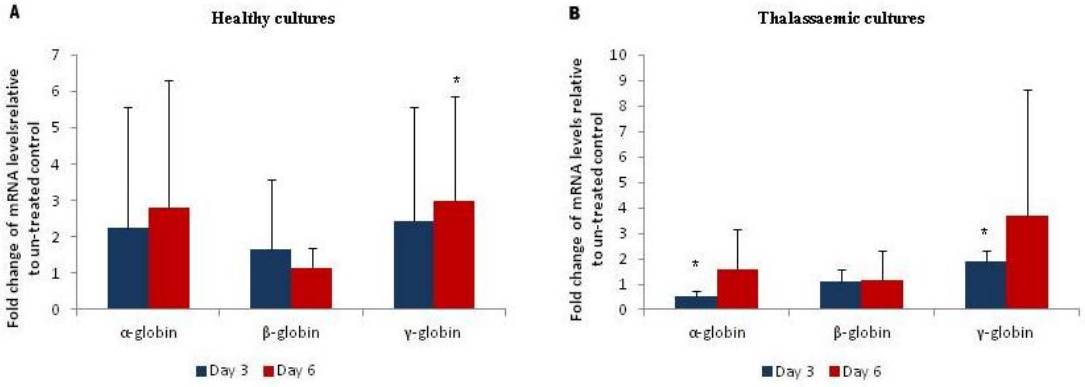
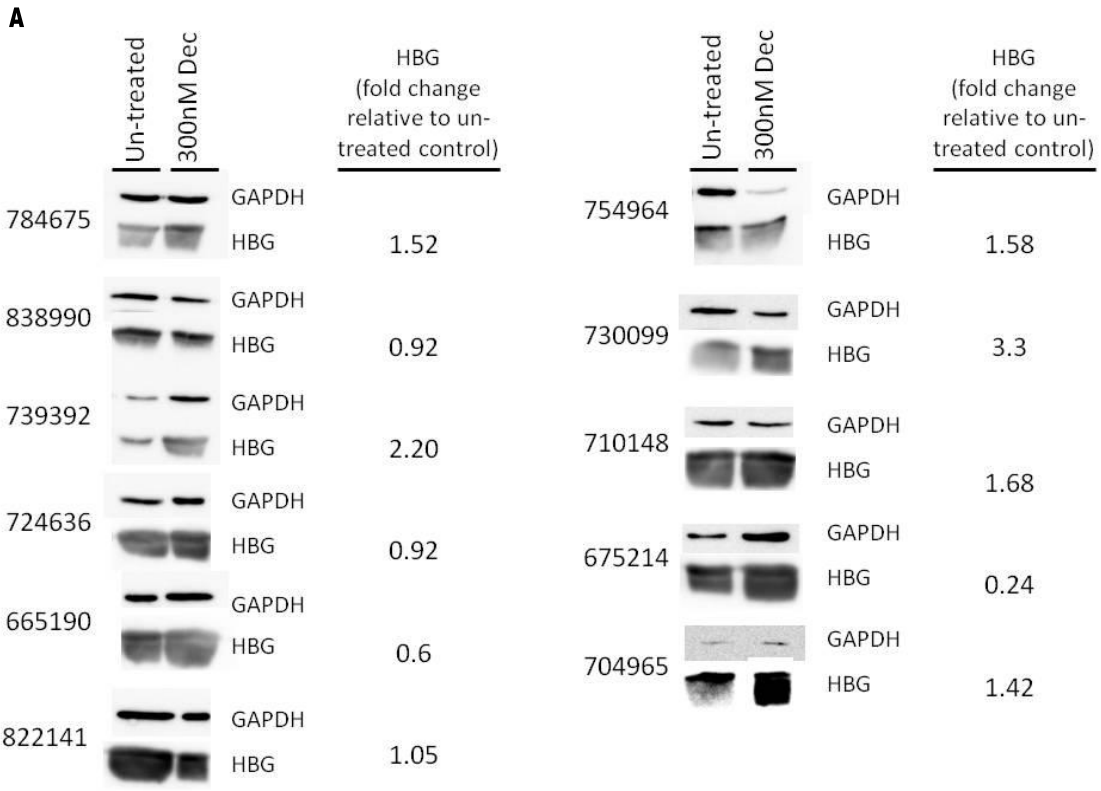
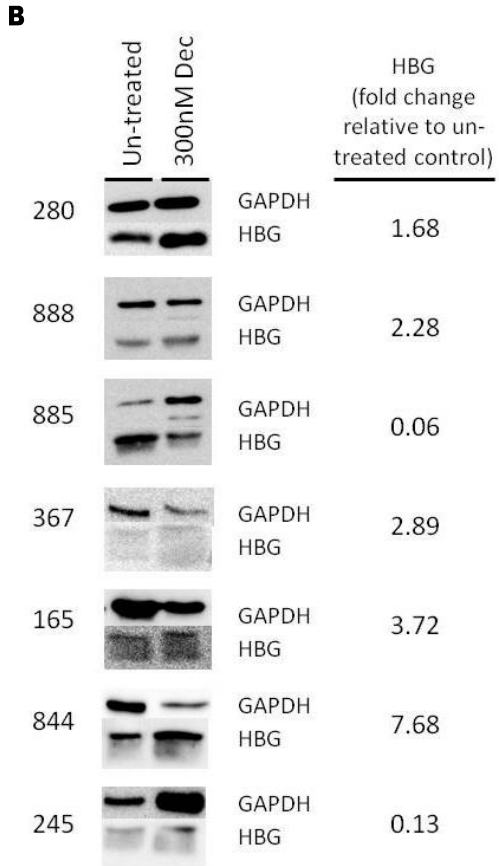


Figure 31, Globin gene expression in primary human erythroid cultures treated with 300nM decitabine. The α-, β- and γ-globin mRNA levels were determined by quantitative real-time PCR after 3 and 6 days of treatment with decitabine in cultures from healthy (A) and thalassaemic (B) donors. The results are the average of twelve healthy cultures and eleven thalassaemic cultures with error bars corresponding to the standard deviation. Statistically significant changes are noted by * as determined by a paired t-test with p-value<0.05.





C

Samples	*HBG expression (Fold change relative to un-treated control)*	*p-value (Paired t-test)*
Healthy	2.63 ± 2.6	0.15
Thalassaemic	1.33 ± 0.833	0.20

Figure 32, Western blot analysis of the primary human erythroid cultures from thalassaemic (A) and healthy (B) donors in the presence and absence of 300nM decitabine (dec). Results demonstrate the levels of HBG along with the endogenous control GAPDH. Quantitation (C) of the results showed an increase in HBG expression both in healthy and thalassaemic cultures in the presence of 300nM decitabine relative to the un-treated control, which was not statistically significant.

3.5.2. Investigation of methylation patterns on globin gene promoters before and after treatment with decitabine

DNA and histone methylation at the promoters of the ε-, γ-, β- and δ-globin genes and the five hypersensitive sites (HS) of the LCR in the β-globin locus (Figure 33A) was analysed by quantitative real-time PCR associated with chromatin immunoprecipitation assay (Section 2.12). Primary human erythroid cultures from three healthy donors were set up according to the previously defined protocol (Section 3.1) and were treated with 300nM decitabine. Confirmation of the effect of the agent in each of the cultures was once more validated by HPLC, in order to ensure that all cultures used for the methylation studies were all responders to decitabine. HPLC analysis (Table 18) showed that all three donors were responsive to the agent with variation in the degree of response.

Table 18, HbF levels of primary human erythroid progenitor cells from healthy donors used for methylation studies. The HbF percentage of the primary human erythroid cultures was determined in the absence and presence of decitabine by HPLC. The table also presents the percentage increase in HbF levels in the presence of the agent.

Healthy donor	***HbF level (%)***		***% increase in HbF***
	Un-treated	***300nM Dec***	
1	4.89	9.29	90.03
2	2.72	5.98	119.85
3	4.12	5.21	26.7

Analysis of histone methylation patterns showed a low Histone 3 lysine 4 dimethylation (H3K4me2) in all globin gene promoters (Figure 33B) in the absence of decitabine. H3K4me2 represents a marker of active chromatin along with H3 Lysine 36 methylation and H3 acetylation (Kiefer *et al.*, 2008) and usually peaks early in active genes (Kim *et al.*, 2007). Among the globin gene promoters, β- and δ-globin promoters had higher H3K4me2 than foetal (γ) and embryonic (ε) globin promoters, suggesting the active expression of adult haemoglobins and the reduced expression of foetal and embryonic haemoglobins. In the LCR region, almost all the HS apart from HS2 show higher H3K4me2 than any of the globin gene promoters in the absence of the drug, suggesting high activity of the LCR (Figure 33B). Following treatment with decitabine, there was a non-significant (p-value>0.05) increase in H3K4me2 levels at the δ-globin

gene promoter, a marked but not significant (p-value>0.05) decrease in H3K4me2 levels at the β-globin gene promoter while H3K4me2 at γ- and ε-globin genes was maintained at approximately the same levels. Interestingly, there was a significant (p-value<0.05) decrease in H3K4me2 in HS3 and HS5 with a marked but not significant (p-value>0.05) increase in HS1 and HS4. H3K4me2 at the ε-γ intergenic region, which served as our negative control, was found to be low both in the absence and presence of the agent as expected (Figure 33B). Based on the above findings it can be suggested that decitabine might affect globin gene expression through modulation of H3K4me2 levels at the HS sites.

Analysis of DNA methylation patterns (Figure 33C) at the globin gene promoters in the absence of decitabine showed that γ-, ε-, β- and δ-globin gene promoters have very low MeCP2 binding, with similar levels to our negative control Cyclin D1. MeCP2 binds to methylated DNA and is thus capable of inhibiting transcription in gene promoters. In the presence of decitabine, the MeCP2 levels dropped substantially at the β-globin gene promoter but remained relatively unchanged at the other globin gene promoters. After treatment with the drug there is a non-significant (p-value>0.05) increase at the HS1 MeCP2 levels and a non-significant (p-value>0.05) decrease at the HS3 and HS5. The findings are controversial with regards to the correlation of DNA methylation and open chromatin since all HS show similar patterns of H3K4m2 (open chromatin) and MeCP2 binding (methylated chromatin) in the presence and absence of decitabine.

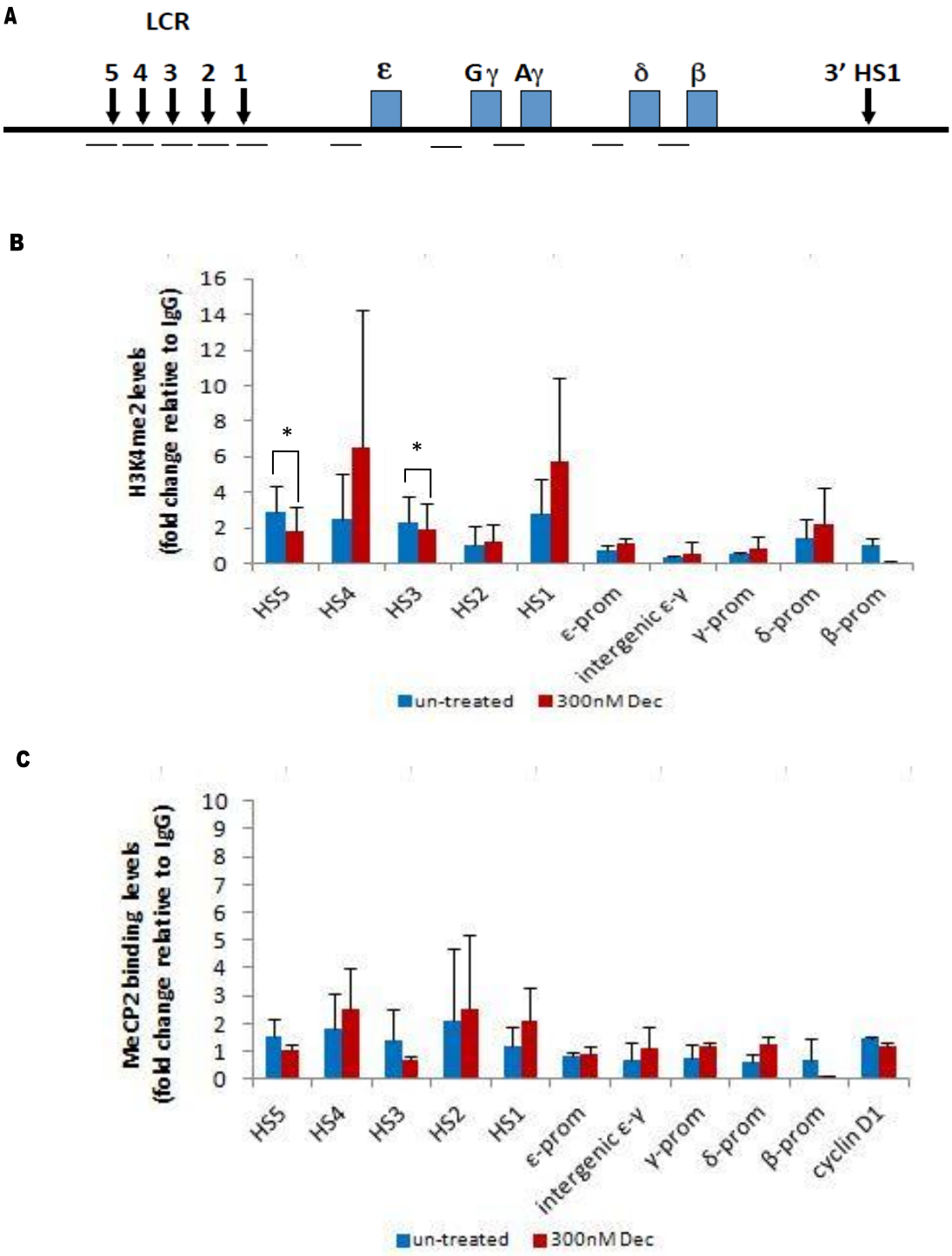


Figure 33, Patterns of Histone and DNA methylation in primary human erythroid cultures from three healthy donors with and without treatment with 300nM decitabine. (A) shows a schematic representation of the β-globin locus and the sites (-) under investigation for methylation status by qRT-PCR assay. H3K4me2 (B) and MeCP2 (C) binding were investigated in all five hypersensitive sites in the LCR and ε-,γ-, β- and δ-globin gene promoters. The results represent the average of three experiments with error bars corresponding to the standard deviation. The MeCP2 results of the β-promoter were the average of two experiments because the third experiment was an extreme outlier and was therefore exluded from calculation. * corresponds to statistically significant changes in methylation patterns according to the paired t-test (p-value<0.05) .

3.5.3. The effect of decitabine on gene expression levels of ten erythroid related genes in primary human erythroid cultures

Primary human erythroid cultures from healthy donors and transfusion-dependent patients set up previously (Section 3.5.1) were used for the investigation of the mRNA levels of ten erythroid-related genes. Changes in the mRNA levels of the above genes were investigated in eleven primary human erythroid cultures from healthy donors and eleven from thalassaemic patients after 3 and 6 days of treatment with 300nM decitabine. The differential expression of the above genes in the absence and presence of decitabine, was investigated by quantitative Real-time PCR (Section 2.10.2). The results were presented as fold changes in gene expression relative to the corresponding un-treated negative controls.

In healthy erythroid cultures, expression of almost all ten genes increased to ~1.5 fold over the un-treated control after 3 days of treatment with decitabine but the expression levels dropped to un-treated equivalents after 6 days with decitabine (Figure 34A). γ-globin gene expression increased by a 2.5 and 3-fold after 3 and 6 days of treatment with decitabine, respectively, relative to the un-treated control (Figure 34A).

In thalassaemic cultures, expression levels of *HBG* (Figure 34B) increased significantly by 2.3-fold (p-value<0.05) above the un-treated control after 3 days of treatment with the agent, and non-significantly by 3.2-fold after 6 days of treatment. In addition, decitabine reduced the expression levels of *BCL11A* to 0.64 that of un-treated control after 3 days of treatment, a difference that was statistically significant (p-value<0.05). After 6 days of treatment with decitabine, expression levels of almost all genes increased relative to the un-treated control. The expression levels of *KLF1, SP1, SOX6* and *CREB* were close to 2-fold while that of *MBD2* approached a 3-fold increase compared to the un-treated control.

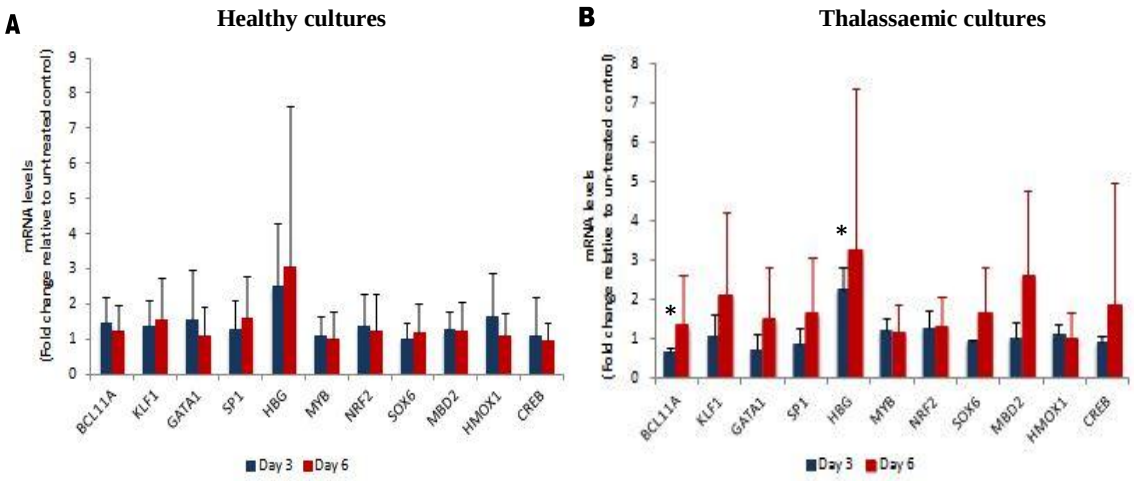
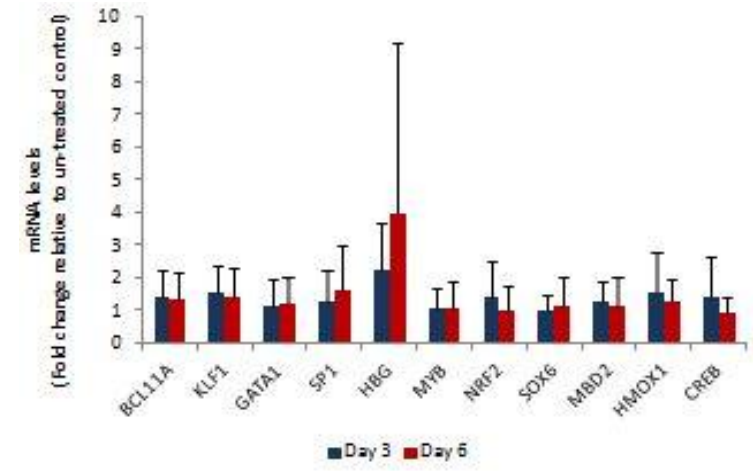


Figure 34, Investigation of the mRNA levels of γ-globin and ten erythroid-related genes in primary human erythroid cultures from eleven healthy (A) and eleven transfusion-dependent thalassaemic donors (B) treated with 300nM decitabine. The results represent the average levels of mRNA after three and six days of treatment with the agent. Error bars correspond to the standard deviation. * corresponds to statistically significant changes in mRNA levels relative to the un-treated control according to the paired t-test (p-value<0.05).

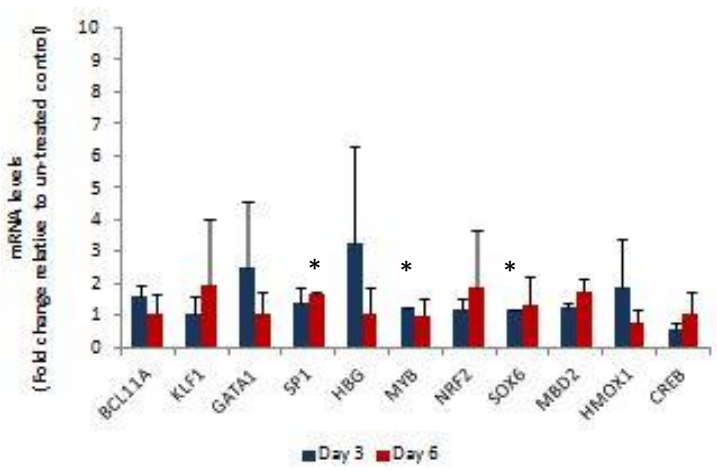
Based on their increase in HbF percentage following treatment with decitabine, primary human erythroid cultures could be grouped into high responders (Figure 35A & C) and low responders (Figure 35B & D). High responders had >50% increase in HbF levels (as measured by HPLC) whereas in low responders, the HbF increase was <50% following treatment with decitabine. There were 8 high responders and 3 low responders for the healthy cultures; and 7 high responders and 4 low responders in the thalassaemic cultures. The values are the average of triplicates from one biological experiment for each culture.

Among the high responders in healthy cultures (Figure 35A) after 3 days of treatment with the agent, the expression of *BCL11A*, *KLF1*, *NRF2*, *HMOX1* and *CREB* increased by ~1.5-fold relative to the un-treated control (Table 19). After 6 days incubation with the agent, expression levels of most of the above genes were at similar levels expect for CREB which showed a moderate but non-significant drop to almost half (Figure 35B, Table 19). In contrast to high responders, the expression of *BCL11A*, *GATA1* and *HMOX1* increased non-significantly to 1.6, 2.48 and 1.86 respectively in low responders after 3 days treatment with decitabine. In addition, the expression of *MYB* and *SOX6* in low responders increased significantly (p-value<0.05) after 3 days of treatment.

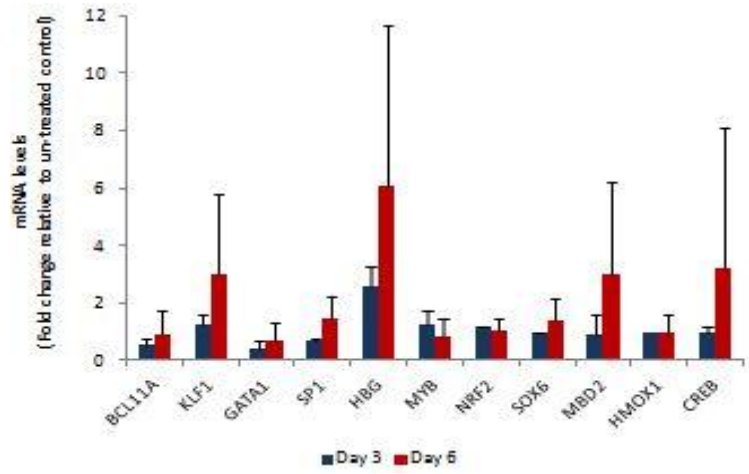
A


Healthy High responders
mRNA levels
(Fold change relative to un-treated control)
BCL11A
KLF1
GATA1
SP1
HBG
MYB
NRF2
SOX6
MBD2
HMOX1
CREB
Day 3
Day 6

B


Healthy Low responders
mRNA levels
(Fold change relative to un-treated control)
BCL11A
KLF1
GATA1
SP1
HBG
MYB
NRF2
SOX6
MBD2
HMOX1
CREB
Day 3
Day 6

C


Thalassaemic High responders
mRNA levels
(Fold change relative to un-treated control)
BCL11A
KLF1
GATA1
SP1
HBG
MYB
NRF2
SOX6
MBD2
HMOX1
CREB
Day 3
Day 6

D

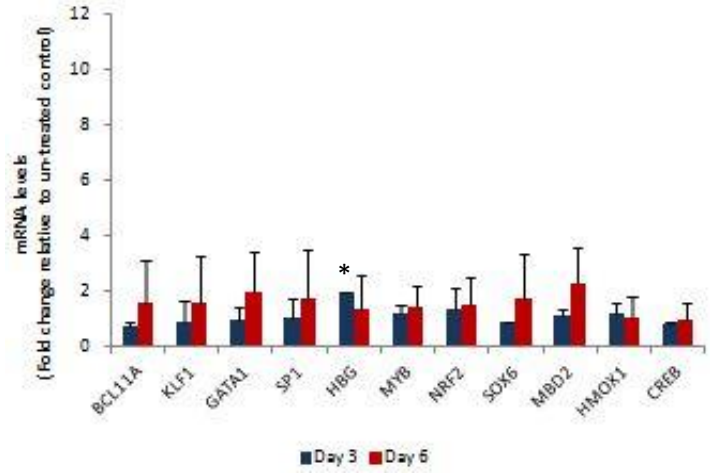

Thalassaemic Low responders
mRNA levels
(Fold change relative to un-treated control)
BCL11A
KLF1
GATA1
SP1
HBG
MYB
NRF2
SOX6
MBD2
HMOX1
CREB
Day 3
Day 6

Figure 35, mRNA levels of γ-globin and ten erythroid-related genes in primary human erythroid cultures from healthy (A+B) and thalassaemic donors (C+D). Primary human erythroid cultures were divided into high responders (A+C) and low responders (B+D) to decitabine based on the increase in HbF percentage determined by HPLC analysis following treatment with decitabine. The results are the average of 2-8 cultures. Error bars correspond to the standard deviation. Statistically significant changes according to the paired t-test (p-value<0.05), are marked with *.

Table 19, mRNA levels of the ten erythroid-related genes in primary human erythroid cultures from healthy (A) and thalassaemic (B) donors in the presence of decitabine. Primary human erythroid cultures were divided into high responders and low responders to decitabine based on the increase in HbF percentage (HPLC) following treatment with decitabine. The values represent the fold change in mRNA level of each gene, as measured by Real-time PCR, in the presence of decitabine relative to the un-treated control. The results are the average of 2-8 cultures ± the standard deviation.

A

Healthy cultures	***Day 3***		***Day 6***	
	High responders	***Low responders***	***High responders***	***Low responders***
Genes	***Average***	***Average***	***Average***	***Average***
BCL11A	1.427 ± 0.827	1.603 ± 0.308	1.313 ± 0.817	1.011 ± 0.666
KLF1	1.533 ± 0.809	1.028 ± 0.534	1.423 ± 0.841	1.975 ± 2/019
GATA1	1.123 ± 0.814	2.475 ± 2.119	1.170 ± 0.866	1.012 ± 0.692
SP1	1.233 ± 1.017	1.406 ± 0.443	1.590 ± 1.42	1.661 ± 0.099
HBG	2.204 ± 1.5	3.267 ± 2.99	3.927 ± 5.288	1.009 ± 0.829
MYB	1.080 ± 0.618	1.252 ± 0.029	1.029 ± 0.863	0.971 ± 0.579
NRF2	1.428 ± 1.098	1.189 ± 0.34	0.994 ± 0.717	1.878 ± 1.77
SOX6	0.958 ± 0.514	1.204 ± 0.0012	1.148 ± 0.886	1.347 ± 0.841
MBD2	1.276 ± 0.624	1.263 ± 0.118	1.144 ± 0.866	1.731 ± 0.395
HMOX1	1.561 ± 1.211	1.861 ± 1.536	1.222 ± 0.695	0.746 ± 0.458
CREB	1.427 ± 1.224	0.534 ± 0.209	0.921 ± 0.498	1.047 ± 0.654

B

Thalassaemic cultures	***Day 3***		***Day 6***	
	High responders	***Low responders***	***High responders***	***Low responders***
Genes	***Average***	***Average***	***Average***	***Average***
BCL11A	0.591 ± 0.1545	0.697 ± 0.1341	0.905 ± 0.819	1.521 ± 1.552
KLF1	1.245 ± 0.363	0.869 ± 0.7891	2.980 ± 2.793	1.542 ± 1.7422
GATA1	0.446 ± 0.236	0.954 ± 0.4651	0.689 ± 0.627	1.908 ± 1.483
SP1	0.708 ± 0.043	1.034 ± 0.6475	1.447 ± 0.758	1.733 ± 1.769
HBG	2.567 ± 0.753	1.906 ± 0.0328	6.076 ± 5.59	1.338 ± 1.2105
MYB	1.252 ± 0.477	1.183 ± 0.2921	0.820 ± 0.677	1.378 ± 0.8012
NRF2	1.157 ± 0.035	1.352 ± 0.7493	1.028 ± 0.470	1.463 ± 1.0321
SOX6	0.966 ± 0	0.870 ± 0.0294	1.423 ± 0.76	1.745 ± 1.576
MBD2	0.896 ± 0.701	1.111 ± 0.1836	2.990 ± 3.219	2.218 ± 1.3304
HMOX1	0.993 ± 0	1.144 ± 0.3876	0.9439 ± 0.629	1.001 ± 0.819
CREB	1.00 ± 0.22	0.819 ± 0.232	3.196 ± 4.915	0.940 ± 0.650

Following 6 days of treatment with decitabine, *HMOX1* and *GATA1* expression in low responders decreased to un-treated levels while that of *KLF1*, *NRF2* and *MBD2* showed a non-significant increase. *SP1* expression was increased significantly (p-value<0.05) in low responders after 6 days of treatment. In low responders of healthy cultures, the increase in *HBG* expression was higher after 3 days of treatment with the agent (Figure 35B), rather than after 6 days of treatment as observed in the high responders (Figure 35A).

In thalassaemic cultures, high responders (Figure 35C) showed a decrease in *BCL11A* and *GATA1* expression to ~0.5-fold after 3 days of treatment, which later increased to un-treated levels after 6 days of treatment for both genes (Table 19). High responders showed a substantial increase in *KLF1*, *MBD2* and *CREB* expression of ~3-fold after 6 days of treatment with decitabine although the increase was not statistically significant (Figure 35C, Table 19). Among the low responders in thalassaemic cultures (Figure 35D), the expression of most genes under investigation apart from *HBG*, was similar to the un-treated levels, after 3 of treatment. After 6 days of treatment with decitabine, low responders showed an increase in *GATA1*, *SP1*, *SOX6* and *MBD2* expression of ~2-fold. Similarly to the cultures from healthy donors, induction of the γ-globin gene in low responders of thalassaemic cultures was higher after 3 days of treatment while in the high responders higher induction was observed after 6 days of treatment with the agent.

Although there is a lack of statistical significance in most of the changes in mRNA levels, some changes are substantial with expression levels changing by 2-fold. The lack of significance might be attributed to the variation in response between individuals.

3.5.4. Discussion

HbF levels in all primary human erythroid cultures increased with increasing length of incubation with the agent in both healthy and thalassaemic cultures as determined by HPLC. The increase in HbF percentage was statistically significant only after 6 days of incubation with the agent. The increase in γ-globin expression was also confirmed by real-time PCR. However, in contrast to HPLC analysis, real-time PCR has shown a greater increase in γ-globin gene expression in thalassaemic compared to that in healthy cultures. A concurrent increase in the α-globin gene expression was noted in healthy but not in thalassaemic cultures after 3 days of treatment suggesting a difference in the response to the agent in the presence of the disease state. Western blot analysis

confirmed the HPLC results, showing that the increase in HbF levels was greater in healthy rather than in thalassaemic cultures. This might be attributed to the high baseline HbF percentage already present in thalassaemic cultures. However, although induction of HbF was lower in thalassaemic cultures, the HbF percentage in the presence of the agent reached levels that might be clinically beneficial.

Despite the general increase in γ-globin expression as demonstrated by all the techniques, a large variation in the response to the agent was observed between individuals. This variation in response was also observed in relation to HU (Pourfarzad *et al.,* 2013). The most common genetic predictor predisposing the beneficial effect by HU was demonstrated to be the baseline HbF level that was associated with the presence of HbF-associated SNPs in *BCL11A* gene or to the XmnI restriction site polymorphism of the γ-globin gene promoter (Ronchi and Ottolenghi, 2013, Stamatoyannopoulos, 2005). Menzel *et al.* (2007) suggested that quantitative trait loci variation in thalassaemia might affect the erythropoietic stress response and thus underlie the variability in clinical severity and capacity of individuals to respond to HbF inducers.

Since decitabine is a hypomethylating agent, the effect of the agent on DNA and histone methylation was investigated in primary human erythroid cultures. Analysis of histone methylation patterns in primary human erythroid cultures demonstrated that apart from HS2 in the β-globin LCR, all HS sites in primary human erythroid cultures from healthy donors show high H3K4me2. Since methylation of H3K4 and H3K36 is associated with open chromatin and gene transcription, higher H3K4me2 at the LCR might support the dependence of transcriptional activation of globin genes on the β-globin LCR. This coincides with the idea that LCR controls the sequential human globin gene expression during development. Kim *et al.* (2007) also showed that the H3K4me2 is present throughout the LCR and at the coding sequence of the transcribed γ-globin gene. H3K4me2 was moderate in the β- and δ-globin gene promoters and minimal in the γ- and ε-globin gene promoters in the absence of induction. This coincides with previous reports that show the δβ domain to be highly enriched for H3K4me2 along with H3K4me3 and H3ac while the εγ domain lacked these histone modifications in adult cells from anaemic spleen of transgenic mice and in primary human erythroid progenitor cells (Miles *et al.,* 2007).

Treatment of primary human erythroid cultures with decitabine resulted in a significant decrease of the H3K4me2 at the HS3 and HS5, with a non-significant increase in H3K4me2 levels at the HS1 and HS4. Following decitabine treatment, small non-significant increases in H3K4me2 at the γ- and δ-globin gene were observed. Similar to our findings, Lavelle *et al.* (2006) demonstrated higher H3K4me2 levels at the β-globin promoter than in the γ-and ε-globin gene promoters in un-treated baboon bone marrow erythroid cells with no significant change in those levels after decitabine treatment. Chin *et al.* (2009) showed that erythroid progenitor cells from the bone marrow of adult baboons with reactivated HbF, had increased levels of H3K4me3 at the γ-globin gene comparable to the levels observed at the β-globin gene, suggesting similar levels of expression of the two genes. In contrast, we have shown a marked decrease in the expression of H3K4me2 at the β-globin promoter. Although not statistically significant, the drop in H3K4me2 levels in the β-globin promoter suggests the decrease in transcription of β-globin by decitabine. This is supported by the small decrease in β-globin mRNA levels after 6 days of treatment with decitabine (Section 3.5.1). A decrease in β-globin expression along with an increase in γ-globin expression was also reported by Mabaera *et al.* (2008a) following treatment of $CD34^+$ cells with 5-azacytidine.

Investigation of DNA methylation in the absence of decitabine showed low levels of MeCP2 binding at all globin gene promoters but high at all five HS sites. Treatment of primary human erythroid cultures with decitabine caused no significant changes in the MeCP2 levels throughout the β-globin locus with the exception of a marked decrease in the MeCP2 levels at the β-globin promoter. Similar to the patterns observed with H3K4m2, decitabine reduced the levels of MeCP2 at the HS3 and HS5 while increasing MeCP2 at HS1. However, these results are in contrast to the function of MeCP2, since a drop of MeCP2 levels at the β-globin promoter would represent a demethylated promoter and active gene while a drop in H3K4m2 would represent reduced transcription of the gene. We suggest that the MeCP2 might act as an activator rather than a repressor of transcription. In fact, studies have shown that gene silencing due to DNA methylation does not depend solely on the binding of MeCP2 (Hendrich and Bird, 1998). It was also suggested that the ability of MeCP2 to repress transcription was dependent upon the density of methyl CpG residues (Nan *et al.,* 1997) which induces assembly of chromatin-condensing secondary structures in the absence of methylation (Georgel *et al.,* 2003). Moreover, Chahrour *et al.* (2008) demonstrated that MeCP2 can

act as both activator and repressor of transcription, with 85% of the genes being activated by MeCP2 in hypothalamus of mice. They further showed that in contrast to repressed genes, gene promoters of MeCP2-activated genes were enriched in CpG islands that were not heavily methylated (Chahrour *et al.*, 2008). It can be therefore concluded that a more specific and accurate approach should have been considered for determination of DNA methylation patterns of globin gene promoters, such as bisulfite sequence analysis.

However, recent studies investigating the potential mechanisms of action of 5-azacytidine suggest that hypomethylation is only a secondary effect of the agent. Mabaera *et al.* (2008a) showed a decrease in γ-globin promoter methylation with no major change in global DNA demethylation in the presence of the agent. In addition, the authors demonstrated that inhibition of DNMT1 expression by RNAi did not induce γ-globin expression sufficiently (Mabaera *et al.*, 2008a). This was confirmed by Chin *et al.* (2009) where they demonstrated that increased γ-globin expression does not correlate with DNA hypomethylation of the γ-globin promoter in decitabine-treated baboons. Based on the above findings, it was suggested that demethylation by 5-azacytidine is a secondary localised effect related to gene activation by other mechanism. It is possible that such a mechanism of action is also relevant to decitabine. In fact, studies have shown that decitabine acts by increasing transcription of γ-globin genes by transcriptional and post-transcriptional processes (Akpan *et al.*, 2010, Chin *et al.*, 2009).

In order to determine whether γ-globin induction by decitabine might be associated with gene activation by other mechanisms, we investigate the differential expression of ten erythroid-related genes after treatment with decitabine in primary human erythroid cultures from healthy and transfusion-dependent thalassaemic donors. Apart from the increase in *HBG* levels following decitabine treatment of primary human erythroid cultures from healthy donors with decitabine, no marked changes were observed in the remaining genes. We grouped the cultures based on their HbF response to decitabine; high responders has >50% increase in the HbF percentage and low responders <50%. In the high responders, there was an increase in *BCL11A*, *KLF1*, *NRF2*, *HMOX1* and *CREB* expression relative to the un-treated control after 3 days of treatment. Changes in the levels of expression of these genes are likely to be downstream effect of decitabine. For examples, the increase in *HMOX1* can be mediated by the increase in NRF2

expression which in turn might induce γ-globin expression. Induction of *HMOX1* was previously shown to be regulated at the transcription level by the stress response element/NRF2 transcription factor pathway (Alam and Cook, 2003). Macari and Lowery (2011) showed that tert-butylhydroquinone increase γ-globin mRNA levels through increased cellular levels of NRF2 and increased binding of the transcription factor to the γ-globin promoter. Recent studies have demonstrated regulation of γ-globin gene expression by p38 MAPK-mediated activation of *CREB* (Ramakrishna and Pace, 2011), thus supporting the increase in CREB expression observed in this study. In contrast, there was a significant increase in *MYB* and *SOX6* expression after 3 days of treatment with decitabine in low responders. After 6 days of treatment, low responders showed a significant increase in *SP1* expression.

In thalassaemic cultures, expression levels of *BCL11A* and *GATA1* are reduced after 3 days of treatment, but their expression levels returned to un-treated levels after 6 days of treatment. High responders of thalassaemic cultures showed a substantial increase in *KLF1, MBD2* and *CREB* expression of ~3-fold after 6 days of treatment with the agent. However, the increase in *KLF1* expression is not consistent with the increase in *HBG* expression. We speculate that its high expression in thalassaemic cultures might be due to the increased expression of *CREB* since CREB can activate KLF1 by direct interaction. Moreover, the increase in *MBD2* gene expression suggests stimulation of methylation within thalassaemic cultures which is not consistent with the function of decitabine as hypomethylating agent but coincides with the effect observed by ChIP analysis that shows increase in methylation pattern at the δ-promoter and HS after treatment with decitabine (Section 3.5.2). In contrast, low responders increased *BCL11A* and *GATA1* expression after 6 days of treatment, and maintained low levels of *CREB.* The increase in *BCL11A* coincides with the low *HBG* expression since *BCL11A* is a silencer of γ-globin gene. Both low and high responders showed an increase in *KLF1*, *SP1* and *MBD2* levels after 6 days of treatment with decitabine.

Based on the above findings, treatment of primary human erythroid cultures with decitabine seems to have a wide effect on gene expression of a number of genes known to be associated with γ-globin regulation. However, most of the changes in gene expression following treatment with decitabine are only subtle and lack statistical significance. This lack of statistical significance might be attributed to the large variation in the response to the agent in the presence of a relatively small sample size. It

is obvious though that the cultured erythroblasts treated with decitabine express γ-globin at much higher levels than any of the other genes tested. Comparing the changes in mRNA levels of genes in high responders of healthy and thalassaemic cultures, it can be observed that *KLF1* levels are the highest in both groups after 6 days of treatment. Whereas, *SP1* and *MBD2* expression are among the highest in low responders of both healthy and thalassaemic cultures after 6 days of induction with the agent. Apart from the above common findings, most changes in the expression levels of the genes investigated were different in healthy and thalassaemic cultures. This suggests that decitabine might act through different mechanisms in healthy and disease environment. However, these are only assumptions and need to be further investigated in a more extensive and detailed transcriptomic study.

3.6. Differential proteomic analysis of primary human erythroid cultures treated with decitabine

3.6.1. Introduction

Emerging evidence suggest that the mRNA expression patterns are not sufficient to describe biological systems. Post-transcriptional mechanisms, controlling the protein translation rate and half-life of proteins, and regulated destruction of proteins that control the turnover of proteins and molecular association of protein products, are among the mechanisms that affect protein abundance. These render the measurement of protein levels essential for analysis of biological processes since proteins are the direct mediators of cellular behaviour. Quantitative proteomics provide a method to measure the relative amounts of proteins in a biological system of interest and to determine how the quantities of those proteins vary in response to particular stimuli such as decitabine.

To further enhance our understanding of how decitabine induces HbF production in primary human erythroid cultures, we compared the proteomes of primary human erythroid cultures before and after decitabine. We adopted an isobaric tag for relative and absolute quantitation (iTRAQ) proteomic approach coupled with mass spectrometry. iTRAQ proteomic approach is a powerful technique for protein expression profiling studies due to its ability to identify and quantitate different proteins simultaneously as well as due to its ability to allow multiplex analysis. Six primary human erythroid cultures from healthy donors and six from thalassaemic patients (Table 20) were selected from Section 3.5.1 for proteomic analysis based on their high increase in the percentage of HbF after both 3 and 6 days of treatment with decitabine, as determined by HPLC analysis.

3.6.2. Quantitative proteomic analysis of primary human erythroid cultures in the presence of decitabine

Proteomic analysis was performed on each of the twelve primary human erythroid cultures individually, in three 8-plex experiments. For each 8-plex experiment, the un-treated and treated (300nM decitabine) samples of two primary human erythroid cultures from healthy subjects (i.e. 4 samples), and from two thalassaemic subjects (i.e. 4 samples) were lysed, labelled and analysed by the Dionex Ultimate 3000 UHPL

system coupled with the high resolution nano-ESI Orbitrap-XL mass spectrometer. Results from the three experiments were then combined resulting in a total of 2188 proteins being detected. Around 61.3% of the proteins (1341 out of 2188) were identified with at least two peptide matches per protein. All proteins were identified with an average of 4.25 matched peptides per protein. Consistent with the values reported in the literature, we observed a protein sequence coverage of 0.25-92% with less than 50% of the proteins having a coverage of at least 10% and 8.5% having sequence coverage of more than 40%. Haemoglobin subunits fell within the 10% category of proteins with high coverage. Both gamma globin proteins had a sequence coverage of 88.4%, β-globin a sequence coverage of 91.16% and α-globin a coverage of 76.6%. Haemoglobin subunit delta, theta, mu and zeta were also detected by the proteomic analysis.

The identified proteins span a wide spectrum of molecular weights (MW), ranging from 5 to 632kDa. Around 14.2% of the proteins identified have a molecular weight of <20kDa while the majority of the proteins (~32%) have a molecular weight between 20 and 40kDa. As expected, the proteins with <20kDa MW have a smaller sequence coverage, on average 25%, due to the reduced number of cleavage sites. As previously shown, the iTRAQ approach can identify proteins at the extreme spectrum of molecular weight, in this case 8.6% of the proteins identified had a MW of >120kDa.

Only 3 (*KLF1*, *MBD2* and *HMOX1*) out of the 10 genes investigated by real-time PCR in primary human erythroid cultures from healthy donors (Section 3.5.3), were detected in the proteomic analysis. KLF1 and MBD2 proteins were identified with a sequence coverage of <10% and a single matched peptide whereas HMOX1 was identified with a sequence coverage of 12% and two unique matched peptides.

In order to understand and interpret these data and to generate testable hypotheses on the systemic response of the proteome to decitabine, the proteins identified have to be further classified and filtered. Quantitation and comparison of the levels of each protein in the different erythroid cultures were presented as four ratios:

(1) The first ratio provided a comparison of the levels of the proteins in the treated healthy cultures versus the levels of the proteins in the un-treated healthy cultures (healthy treated/healthy un-treated cultures)

(2) the second ratio was a comparison of the protein levels in the treated thalassaemic cultures versus those in the un-treated thalassaemic cultures (thalassaemic treated/thalassaemic un-treated cultures)

(3) the third ratio compared protein levels in the un-treated thalassaemic cultures versus those in the un-treated healthy cultures (thalassaemic un-treated/healthy un-treated cultures)

(4) the final ratio was a comparison of protein levels in the treated thalassaemic cultures versus those in the treated healthy samples (thalassaemic treated/healthy treated cultures)

Table 20, Data on the six primary human erythroid cultures from healthy donors and six primary human erythroid cultures from thalassaemic patients selected for proteomic analysis. For each culture, the HbF levels before and after 6 days of treatment with decitabine were determined by HPLC analysis. The table shows the HbF percentage before and after treatment for each culture along with the percentage increase in HbF in the presence of decitabine.

Healthy samples				
culture ID	***HbF (%)***		***Increase in HbF (% relative to un-treated)***	***iTRAQ Experiment No***
	un-treated	***300nM Dec***		
974	3.74	8.01	114.20	1
965	10.30	19.65	90.89	1
245	5.65	8.86	56.83	2
885	12.83	19.73	53.78	2
280	5.07	9.1	79.51	3
367	2.53	4.22	66.78	3
Average	*6.69*	*11.60*	*77.00*	

Thalassaemic samples				
culture ID	***HbF (%)***		***Increase in HbF (% relative to un-treated)***	***iTRAQ Experiment No***
	un-treated	***300nM Dec***		
754676	21.79	31.35	43.88	1
784676	15.80	22.65	43.35	1
710148	18.11	45.21	149.68	2
724636	23.48	36.67	56.13	2
838990	9.87	15.46	56.64	3
739392	17.61	27.00	53.29	3
Average	*17.78*	*29.72*	*67.16*	

As expected, the expression levels of most proteins did not change in the presence of decitabine. For the majority of proteins, the values of all four ratios were around 1, with only ≤5% of the proteins being up- or down-regulated by more than 1.5-fold. In order to determine if the proteins are significantly differentially expressed, the ratios were

transformed into log_2 values for each protein in order to normalise the negatively skewed distribution of the proteins for accurate statistical analysis. The proteins within each ratio were then sorted based on their log_2 value and their p-values were calculated. As expected, the p-value distribution of the log_2 values within each ratio is positively skewed towards a p-value of 1 (Figure 36) confirming once more that the expression levels of the majority proteins did not change in the presence of decitabine. A p-value of <0.05 for each log_2 value was used as a cut-off threshold to select the significantly differentially expressed proteins for further analysis. Applying the cut-off threshold narrowed down the differentially expressed proteins to 105 proteins for the healthy treated/healthy un-treated ratio, 110 proteins for the thalassaemic treated/thalassaemic un-treated ratio, 66 proteins for the thalassaemic un-treated/healthy un-treated and 98 proteins for the thalassaemic treated/healthy treated ratio (Appendix IV).

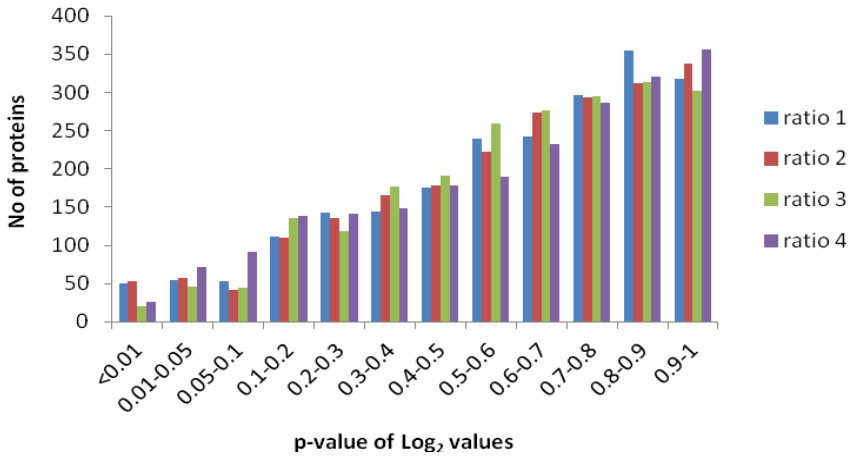


Figure 36, The p-value distribution of the log_2 values of the 2188 proteins identified by proteomic analysis. The expression levels of each protein within each ratio was transformed into log_2 value with a corresponding p-value. Using a p-value<0.05 as a threshold, only a minority of the proteins identified are shown to be significantly differentially expressed within each ratio.

3.6.2.1. Ratio 1- Healthy cultures: Treated versus Un-treated

Among the 105 significantly differentially expressed proteins (p-value<0.05) in healthy cultures (ratio 1), 47 proteins were up-regulated in the presence of decitabine, 95.7% of them with an average expression ratio of over 1.2 and 4.3% with an average expression ratio of over 2 (Appendix IV). 58 proteins were significantly down-regulated by less than two fold, i.e. with an expression ratio of more than 0.5 in the presence of decitabine. Bioinformatics analysis by DAVID (Figure 37A) and GeneCodis (Figure 37B), demonstrated that the significantly differentially expressed proteins in healthy cultures are involved in transcription factor binding, regulation of cell proliferation, protein stabilization, chromatin organization and protein complex assembly. In addition, four differentially expressed proteins were associated with regulation of NF-κB signalling pathway. Looking into the function of the up-regulated and down-regulated proteins directly, it can be suggested that decitabine might affect caspase-mediated apoptotic pathways and ubiquitin ligase complexes due to activation of oxidative or ER stress (Figure 38). This can be supported by the up-regulation of proteins like PYCARD, a mediator of caspase 8 and 9-mediated apoptosis (Hasegawa *et al.*, 2007), COPS2, a regulator of cullin-based ubiquitin E3 ligase complexes that mediates p53 degradation (Bech-Otschir *et al.*, 2001), DNAJA3, modulator of apoptotic signal transduction (Syken *et al.*, 1999) and HTATIP2, a tumor suppressor involved in cellular oxidative stress surveillance which induces p53-mediated apoptosis (Lee *et al.*, 2012, Zhao *et al.*, 2008). Transcriptional inhibitors, including DR1, a TATA-binding protein-associated phosphoprotein inhibitor of basal and activated gene transcription (Inostroza *et al.*, 1992), CNOT8, a subunit of CCR4-NOT complex which regulates RNA polymerase II transcription (Aslam *et al.*, 2009, Kruk *et al.*, 2011) and HEXIM1, an inhibitor of positive transcription elongation factor (Lew *et al.*, 2013), were down-regulated. At the same time proteins that promote transcription such as MYBL1 (Ma and Calabretta, 1994) are up-regulated. These findings support the positive regulation of transcription by decitabine (Figure 38).

A

	Functional clustering	*Enrichment score*	*Count*	*P-value*	*Benjamini*
transcription factor binding	MF	1.56	11	0.0045	0.7500
regulation of cell growth	BP	1.4	5	0.0081	0.9400
regulation of cellular component size	BP	1.4	7	0.0150	0.8900
regulation of protein localisation	BP	1.32	5	0.0120	0.8800
lipid transport	BP	1.32	6	0.0027	0.9400
focal adhesion	CC	1.13	5	0.0200	0.7400
kinase inhibitor activity	MF	0.89	3	0.0680	0.9100
protein stabilization	BP	0.92	4	0.0092	0.9100
protein kinase cascade	BP	0.79	6	0.0350	0.9800
chromatin organization	BP	0.74	8	0.0470	0.9600
transcription cofactor activation	MF	0.72	8	0.0120	0.8300
macromolecular complex assembly	BP	0.7	15	0.0380	0.9700
regulation of I-kappaB kinase/NF-kappaB cascade	BP	0.66	4	0.0550	0.9700
regulation of transferase activity	BP	0.61	6	0.0390	0.9600

B

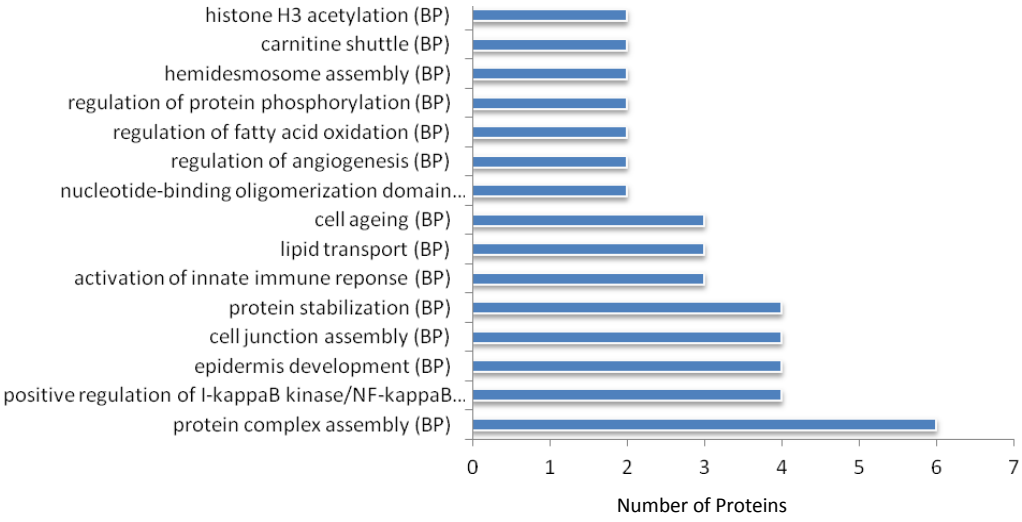


Figure 37, Bioinformatic analysis of the 105 significantly differentially expressed proteins in primary human erythroid cultures from healthy donors treated with decitabine (ratio 1). Functional analysis by DAVID (A) and GeneCodis (B) of significantly differentially expressed proteins (ratios with p-value<0.05) using a reference list of all 2188 proteins identified, grouped proteins according to their biological process (BP), molecular function (MF) and cellular component (CC). In DAVID, protein groups are categorised according to their enrichment scores and corresponding p-value and Benjamini values.

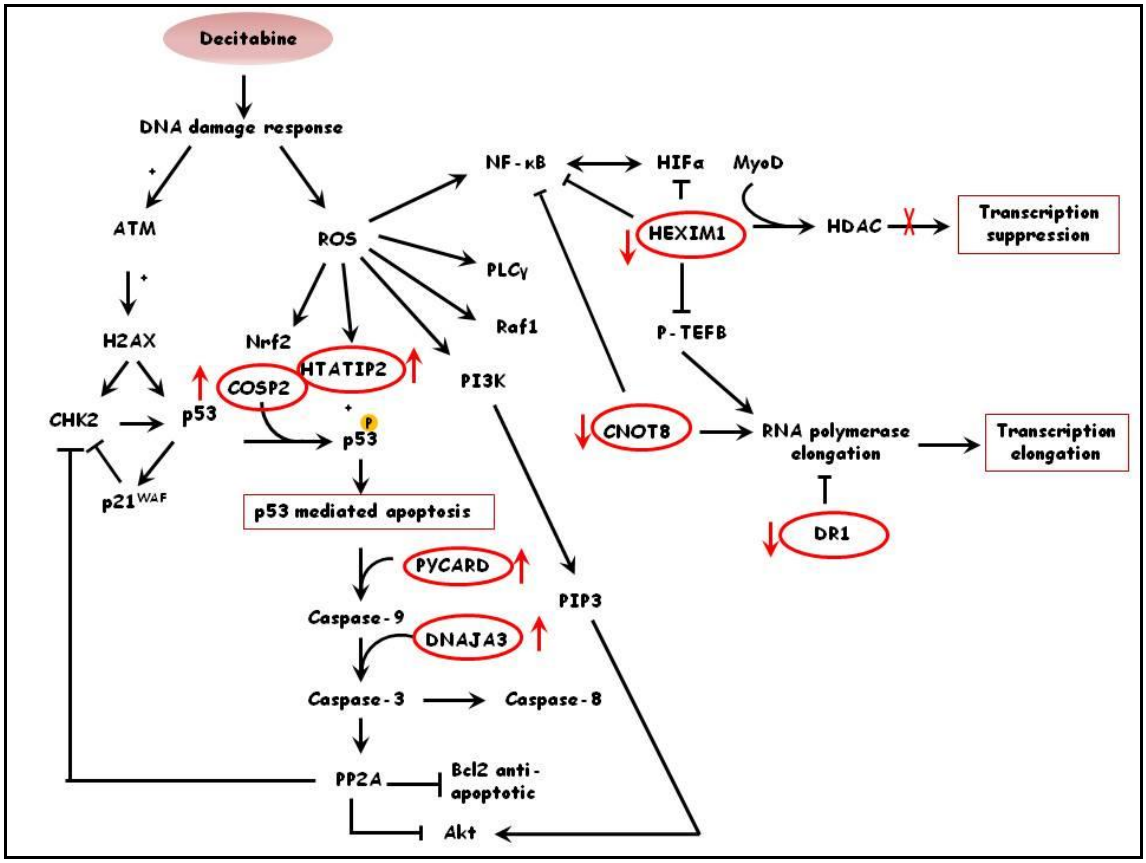


Figure 38, Schematic diagram demonstrating the possible effect of decitabine in primary human erythroid progenitor cells from healthy donors. Decitabine promotes apoptosis through activation of stress responses, and increases transcriptional activity by down-regulation of transcriptional repressors. Proteins in red circle are some of the differentially expressed proteins identified in ratio 1 (i.e. healthy treated/healthy un-treated cultures). The red arrows demonstrated whether the encircled proteins are up- or down-regulated by decitabine.

3.6.2.2. Ratio 2 – Thalassaemic cultures: treated versus un-treated

The comparison of proteins in treated versus un-treated thalassaemic cultures (ratio 2), indicated that 54.5% of the 110 significantly differentially expressed proteins (p-value<0.05) were up-regulated (Appendix IV); by less than 1.2-fold in 45.5%; by a ratio of greater than 1.2-fold in 25.5% and by more than 2-fold in 9% of the proteins. Among the significantly down-regulated proteins, 92.7% are down-regulated by more than 1.4 times while the remaining proteins are down-regulated by more than 2-fold (expression ratio <0.5) (Appendix IV). Functional annotation analysis of the differentially expressed proteins (p-value<0.05) in thalassaemic cultures (Figure 39) showed that the differentially expressed proteins in the presence of decitabine are involved in protein and metal ion binding, DNA binding and chromatin modifications, transcription and positive regulation of NF-κB cascade. Functional clustering of differentially expressed

proteins, as well as looking into the function of individual proteins directly, suggested that decitabine favours production of immature erythrocytes (Figure 40) as can be seen by the down-regulation of proteins involved in erythroid maturation such as PICALM, a clathrin assembly lymphoid myeloid leukemia protein involved in erythroid maturation and transferin internalization in mice (Suzuki *et al.*, 2012), ABCB6, a glycoprotein expressed in the membrane of mature erythrocytes and in exosomes released from reticulocytes at the final steps of erythroid maturation (Kiss *et al.*, 2012) and up-regulation of TACC3, a protein highly expressed in haematopoietic progenitors (Garriga-Canut and Orkin, 2004). Moreover, results suggest that decitabine affects transcriptional activation of genes downstream of oxidative stress pathways such as HMOX1, a target gene of the central regulator of cellular oxidative stress response Nrf2 (Fer *et al.*, 2010) and up-regulation of THOC5, a key protein in the maintenance of haematopoietic stem cells which is phosphorylated in the presence of oxidative stress (Griaud *et al.*, 2013), and PPP5C, a negative regulator of hypoxia-induced activation of apoptosis signal-regulating kinase 1 (Morita *et al.*, 2001) (Figure 40). Proteins such as RBM10, an RNA binding regulator of splicing (Wang *et al.*, 2013), XRN2, a 5'-3' exonuclease that promotes transcriptional termination (Kaneko *et al.*, 2007, West *et al.*, 2004), were up-regulated while HEXIM, a regulator of transcriptional elongation of RNA polymerase II (Lew *et al.*, 2013) was down-regulated. These findings suggest that decitabine promotes transcriptional termination and post-transcriptional processes (Figure 40). Decitabine also appears to regulate chromatin remodelling as can be observed by the up-regulation of SMARCD3, a subunit of SWI/SNF complex that promotes DNA-histone dissociation (Forcales *et al.*, 2012), and down-regulation of CHD5, a chromatin remodelling, helicase and DNA-binding protein which is activated by demethylation of its promoter (Zhao *et al.*, 2014) (Figure 40).

A

	Functional clustering	*Enrichment score*	*Counts*	*P-value*	*Benjamini*
transition metal ion binding	MF	1.52	19	1.70E-02	9.90E-01
zinc finger	MF	1.52	10	2.00E-02	9.80E-01
cerebral cortex development	BP	1.35	3	2.50E-02	9.80E-01
chromatin organization	BP	1.35	12	2.00E-04	1.70E-01
transcription	BP	1.29	14	2.10E-02	9.90E-01
gamete generation	BP	1.13	6	4.10E-02	9.50E-01
chromatin modification	BP	0.98	6	2.30E-02	9.90E-01
positive regulation of I-kappaB kinase/NF-kappaB cascade	BP	0.95	4	3.60E-02	9.50E-01

B

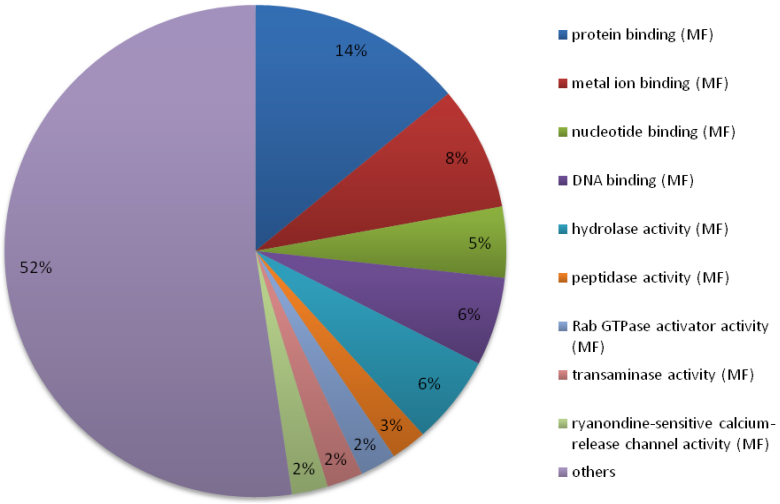


Figure 39, Functional annotation of the 110 significantly differentially expressed proteins in primary human erythroid cultures from thalassaemic donors treated with decitabine (ratio 2). Functional analysis by DAVID (A) and GeneCodis (B) of the significantly differentially expressed proteins (ratios with p-value<0.05) using a reference list of all 2188 proteins identified, grouped proteins according to their biological process (BP), molecular function (MF) and cellular component (CC). In DAVID, protein groups are categorised according to their enrichment scores and corresponding p-value and Benjamini values.

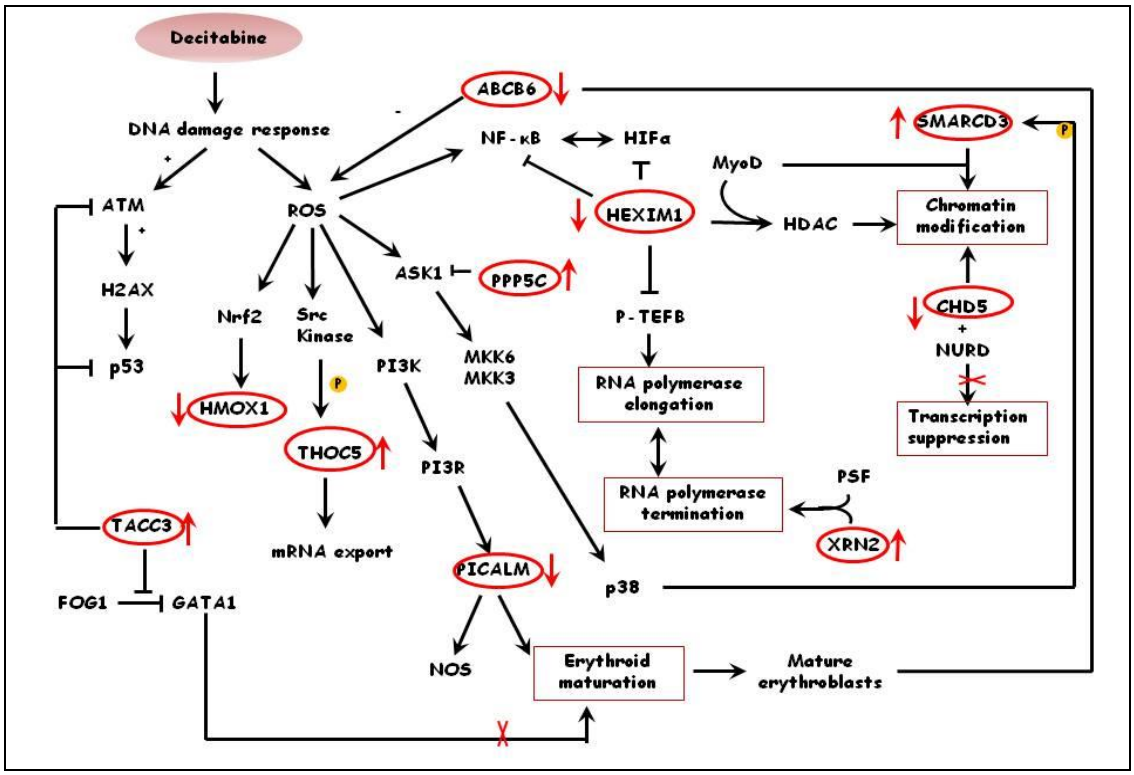


Figure 40, Schematic diagram demonstrating the possible effects of decitabine on primary human erythroid progenitor cells from thalassaemic donors. Decitabine promotes transcription through down-regulation of transcriptional repressors and through chromatin modifications, rather than activation of oxidative stress-induced pathways. In addition, decitabine favours immature erythroblasts and protects them against stresses. Proteins in red circle are some of the differentially expressed proteins in thalassaemic cultures treated with decitabine (ratio 2). The red arrows demonstrated whether the encircled proteins are up- or down-regulated by decitabine.

3.6.2.3. Ratio 3 – Thalassaemic un-treated versus healthy un-treated

Analysis of the ratio of proteins of un-treated thalassaemic samples over un-treated healthy samples (ratio 3) resulted in the identification of 66 significantly differentially expressed proteins (p-value<0.05), 36.3% of which are up-regulated. 87.5% of the up-regulated proteins were expressed with a ratio greater than 2 while the remaining proteins were expressed with a ratio of 1.5 to 2. Among the down-regulated proteins, 57% were down-regulated by more than 1.6 fold and the remaining were down regulated by more than 2 fold (Appendix IV). Differentially expressed proteins were found to be involved in steroid metabolic processes, defence responses, metal ion transport, oxidation-reduction processes and haematopoiesis as shown by the functional clustering (Figure 41); processes that are commonly associated with thalassaemia.

A

	Functional clustering	*Enrichment score*	*Count*	*P-value*	*Benjamini*
Glycoprotein	Keyword	3.19	17	1.10E-04	2.40E-02
extracellular region	CC	3.19	11	3.50E-03	4.10E-01
steroid metabolic process	BP	1.62	4	2.40E-02	9.80E-01
defence response	BP	1.19	7	1.00E-02	1.00E+00
acute-phase response	BP	1.19	3	1.90E-02	1.00E+00
metal ion transport	BP	1.13	4	4.10E-02	9.90E-01
tyrosine protein kinase	MF	0.49	4	5.60E-03	7.00E-01

B

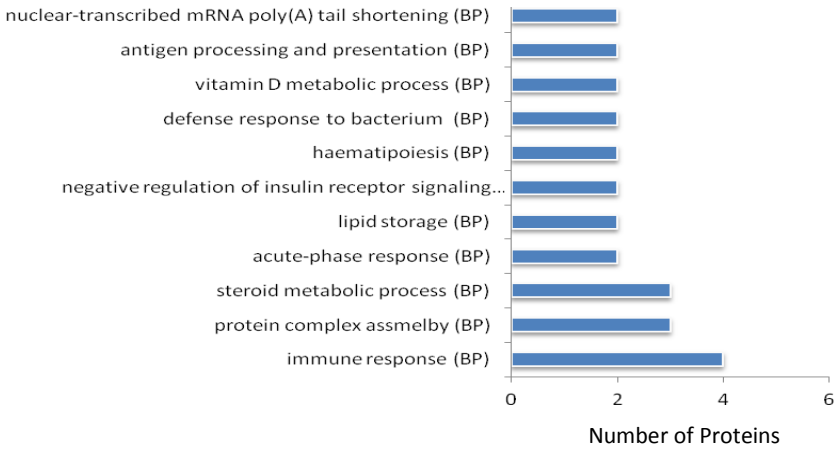


Figure 41, Functional annotation of the 66 significantly differentially expressed proteins of un-treated thalassaemic samples over un-treated healthy samples (ratio 3). Functional analysis by DAVID (A) and GeneCodis (B) of the significantly differentially expressed proteins (ratios with p-value<0.05) using a reference list of all 2188 proteins identified, grouped proteins according to their biological process (BP), molecular function (MF) and cellular component (CC). In DAVID, protein groups are categorised according to their enrichment scores and corresponding p-value and Benjamini values.

3.6.2.4. Ratio 4 – Thalassaemic treated versus healthy treated

98 proteins were found to be significantly differentially expressed when comparing thalassaemic and healthy cultures treated with decitabine (ratio 4) (Appendix IV). 50% of the significantly differentially expressed proteins (p-value<0.05) were up-regulated, 85.7% of which had an expression ratio of greater than 1.2 while 4.3% had a ratio of greater than 2. Among the significantly down-regulated proteins, 57% were reduced by less than 2 fold, while the remaining 43% were reduced by more than 2-fold. Functional clustering of differentially expressed proteins (Figure 42), demonstrated that the differentially expressed proteins are involved in lipid transport, platelet activation and degranulation, immune defence and cell adhesion.

A

	Functional clustering	*Enrichment score*	*Count*	*P-value*	*Benjamini*
Glycoprotein	keyword	4.51	7	2.70E-04	2.20E-01
lipid transport	BP	1.72	6	1.80E-03	5.60E-01
sex differentiation	BP	1.42	5	1.40E-02	9.60E-01
integral to plasma membrane	CC	1.34	20	2.80E-02	5.50E-01
defence response	BP	1.22	8	2.20E-02	9.90E-01
ion transport	BP	0.86	8	3.60E-02	9.90E-01

B

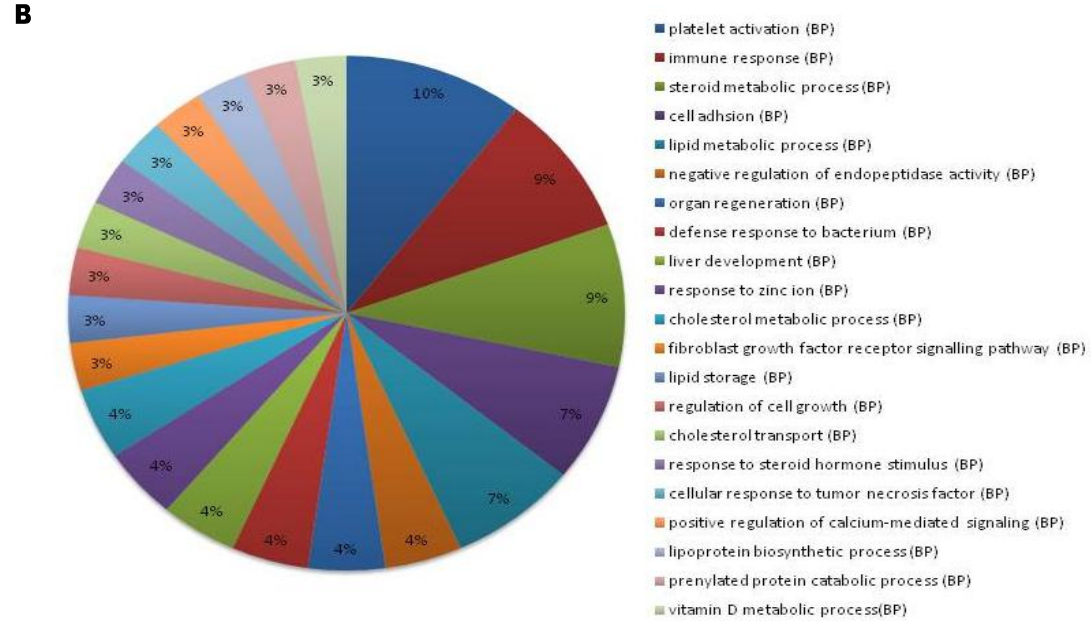


Figure 42, Functional annotation of the 98 significantly differentially expressed proteins in thalassaemic cultures treated with decitabine over healthy cultures treated with decitabine (ratio 4). Functional analysis by DAVID (A) and GeneCodis (B) of the significantly differentially expressed proteins (ratios with p-value<0.05) using a reference list of all 2188 proteins identifed, grouped proteins according to their biological process (BP), molecular function (MF) and cellular component (CC). In DAVID, protein groups are categorised according to their enrichment scores and corresponding p-value and Benjamini values.

Comparison of the functional clusterings of the differentially expressed proteins of each ratio (Table 21), demonstrate that differentially expressed proteins of ratio 1 and 2 fall commonly into three functional annotations: chromatin organization and modification, transcription and regulation of the NF-κB cascade. Differentially expressed proteins of ratio 3 and 4 are commonly involved with steroid metabolism, defence response, haematopoiesis and vitamin D metabolism; biological processes that are involved with the disease state.

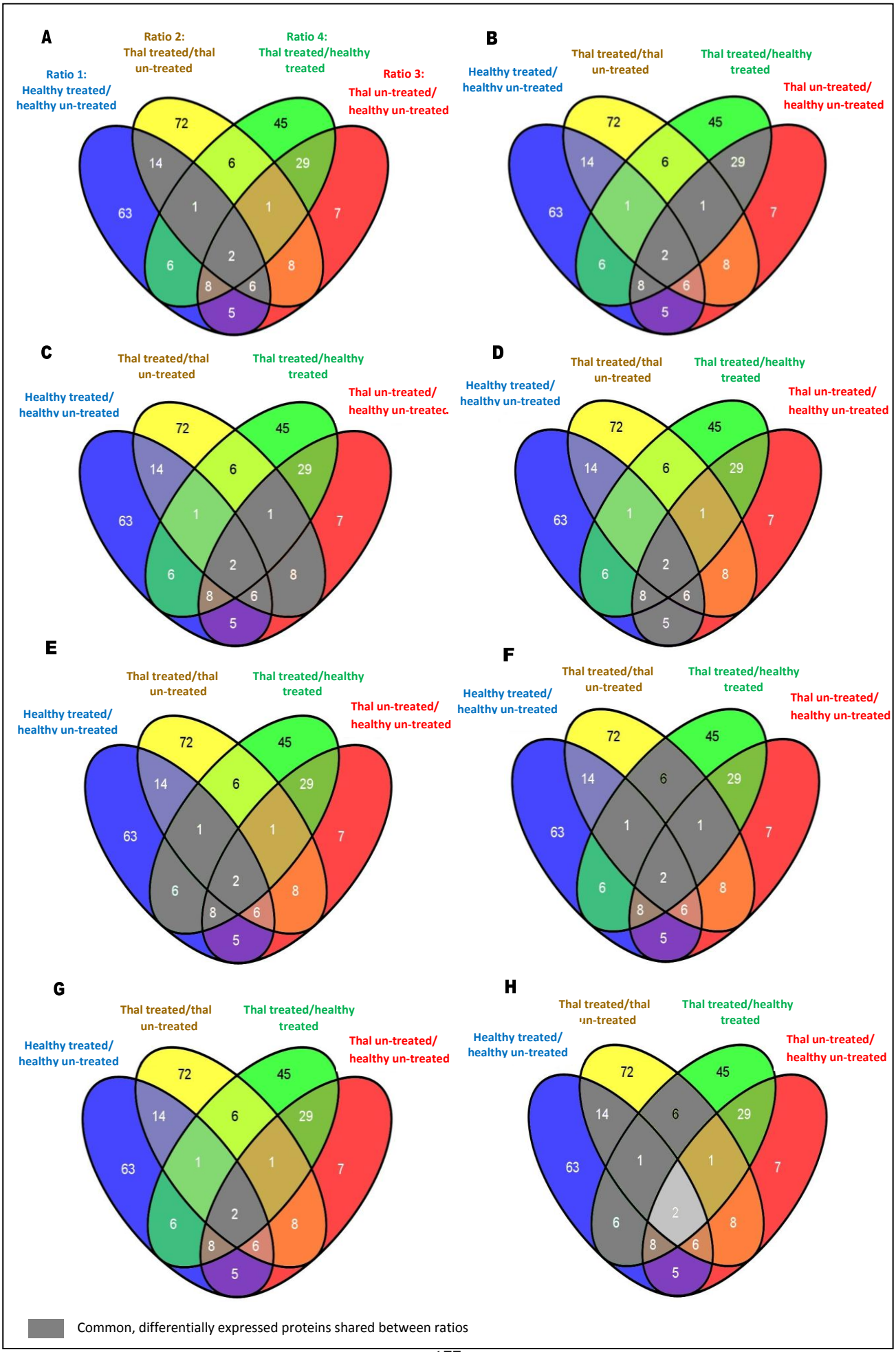
Table 21, Comparison of the functional clustering of the significantly differentially expressed proteins of each ratio. Functional analysis by DAVID and GeneCodis, categorised the differentially expressed proteins of each ratio (ratios with p-value<0.05) according to their Biological process (BP), Molecular function (MF) and cellular component (CC). The table shows the number of differentially expressed proteins within each ratio that belong to each functional annotation.

	Functional clustering	*Ratio 1 (No of proteins)*	*Ratio 2 (No of proteins)*	*Ratio 3 (No of proteins)*	*Ratio 4 (No of proteins)*
regulation of cell growth	BP	5			2
regulation of cellular component size	BP	7			
regulation of protein localisation	BP	5			
protein stabilization	BP	4			
protein complex assembly	BP	15		3	
chromatin organization	BP	8	12		
chromatin modification	BP		6		
transcription	BP	8	14		
lipid transport	BP	6			6
lipid metabolic process	BP				5
fatty acid oxidation	BP	2			
protein kinase cascade	BP	6		4	
regulation of transferase activity	BP	6			
regulation of I-kappaB kinase/NF-kappaB cascade	BP	4	4		
gamete generation	BP		6		5
cerebral cortex development	BP		3		
steroid metabolic process	BP			4	6
defense response	BP	3		7	8
acute-phase response	BP			3	
metal ion transport	BP			4	
hematopoiesis	BP			2	8
vitamin D metabolic process	BP			2	2
platelet activation	BP				7
cell adhesion	BP				5
transcription factor binding	MF	11			
kinase inhibitor activity	MF	3			
DNA binding	MF		5		
transition metal ion binding	MF		19		
zinc finger	MF		10		
glycoprotein	keyword			17	7
extracellular region	CC		3	11	
nucleosome	CC		5		
lysosome	CC			5	
focal adhesion	CC	5			

In order to narrow down the list of differentially expressed proteins to those that are solely associated with the function of decitabine and not due to the disease state, we further compared the four sets of significantly differentially expressed proteins between the four ratios (ratio 1: healthy treated/healthy un-treated samples, ratio 2: thalassaemic treated/thalassaemic un-treated cultures, ratio 3: thalassaemic un-treated/healthy un-treated cultures and ratio 4: thalassaemic treated/healthy treated cultures). Comparison of ratio 1 and 2 indicated 23 common, differentially expressed proteins (Figure 43A, gray area). Comparison of proteins derived from ratio 3 and ratio 4 identified 40

proteins that were commonly differentially expressed (Figure 43B, gray area). Comparison of proteins derived from ratio 2 and 3, identified 17 common, differentially expressed proteins (Figure 43C, gray area). 21 protein were common when comparing differentially expressed proteins in ratios 1 and 3 (Figure 43D, gray area). 17 proteins were commonly differentially expressed when comparing proteins in ratios 1 and 4 (i.e. healthy treated/healthy un-treated samples and thalassaemic treated/healthy treated samples) (Figure 43E, gray area). 10 commonly, differentially expressed proteins were identified when comparing ratios 2 and 4 (i.e. thalassaemic treated/thalassaemic un-treated cultures and thalassaemic treated/healthy treated samples (Figure 43F, gray area) and 2 proteins were commonly differentially expressed among all of the four ratios (Figure 43G, gray area).

Differentially expressed proteins following treatment with decitabine in healthy (105 proteins) and thalassaemic (110 proteins) cultures represent proteins whose expression levels change in the presence of decitabine. In contrast, proteins that are associated with the disease state are proteins whose expression differs between thalassaemic and healthy cultures in the absence of the agent (i.e. proteins in the ratio thalassaemic un-treated/healthy un-treated samples, ratio 3). However, proteins that are shown to be differentially expressed by the ratio of thalassaemic treated over healthy treated samples (ratio 4) but not by the ratio of thalassaemic un-treated/healthy un-treated samples (ratio 3) might include proteins that are up- or down-regulated at higher levels in thalassaemic compared to healthy cultures due to the agent. Therefore, in order to identify the proteins that are solely associated with the presence of decitabine (Figure 44), proteins that were attributed to the disease state were removed from the list of commonly differentially expressed proteins between the treated over un-treated ratios of healthy and thalassaemic cultures. Among the 23 common proteins between thalassaemic and healthy cultures whose expression is altered in the presence of decitabine (Figure 43A and 44), 8 proteins associated with the disease state were removed (Figure 44), resulting in 15 proteins that were solely attributed to the presence of decitabine. 6 proteins that were differentially expressed in thalassaemic cultures due to the presence of decitabine (thalassaemic treated/thalassaemic un-treated cultures, ratio 2) but were also differentially expressed in thalassaemic treated over healthy treated cultures (ratio 4) can be considered as proteins being affected by the agent, since they might represent proteins that are affected more in thalassaemic rather than in healthy cultures following decitabine treatment.


A
Ratio 1:
Healthy treated/
healthy un-treated
Ratio 2:
Thal treated/thal
un-treated
Ratio 4:
Thal treated/healthy
treated
Ratio 3:
Thal un-treated/
healthy un-treated
72
45
14
6
29
63
1
1
7
6
2
8
8
6
5
B
Healthy treated/
healthy un-treated
Thal treated/thal
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Thal un-treated/
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Healthy treated/
healthy un-treated
Thal treated/thal
un-treated
Thal treated/healthy
treated
Thal un-treated/
healthy un-treated
Common, differentially expressed proteins shared between ratios

Figure 43, Venn diagrams representing overlaps of significantly expressed proteins among the four ratios. (A) common proteins between treated/un-treated ratio in healthy (ratio 1) and thalassaemic (ratio 2) cultures. (B) common proteins between thalassaemic un-treated/healthy un-treated ratio (ratio 3) and thalassaemic treated/healthy treated ratio (ratio 4). (C) common proteins of thalassaemic treated/thalassaemic un-treated ratio (ratio 2) and thalassaemic un-treated/healthy un-treated ratio (ratio 3). (D) common proteins between healthy treated/healthy un-treated ratio (ratio 1) and thalassaemic un-treated/healthy un-treated ratio (ratio 3). (E) common proteins between healthy treated/healthy un-treated ratio (ratio 1) and thalassaemic treated/healthy treated ratio (ratio 4). (F) common proteins between thalassaemic treated/thalassaemic un-treated ratio (ratio 2) and thalassaemic treated/healthy treated ratio (ratio 4). (G) common proteins between all four ratios, (H) proteins that are differentially expressed in response to decitabine. Grey highlighted areas represent the common proteins

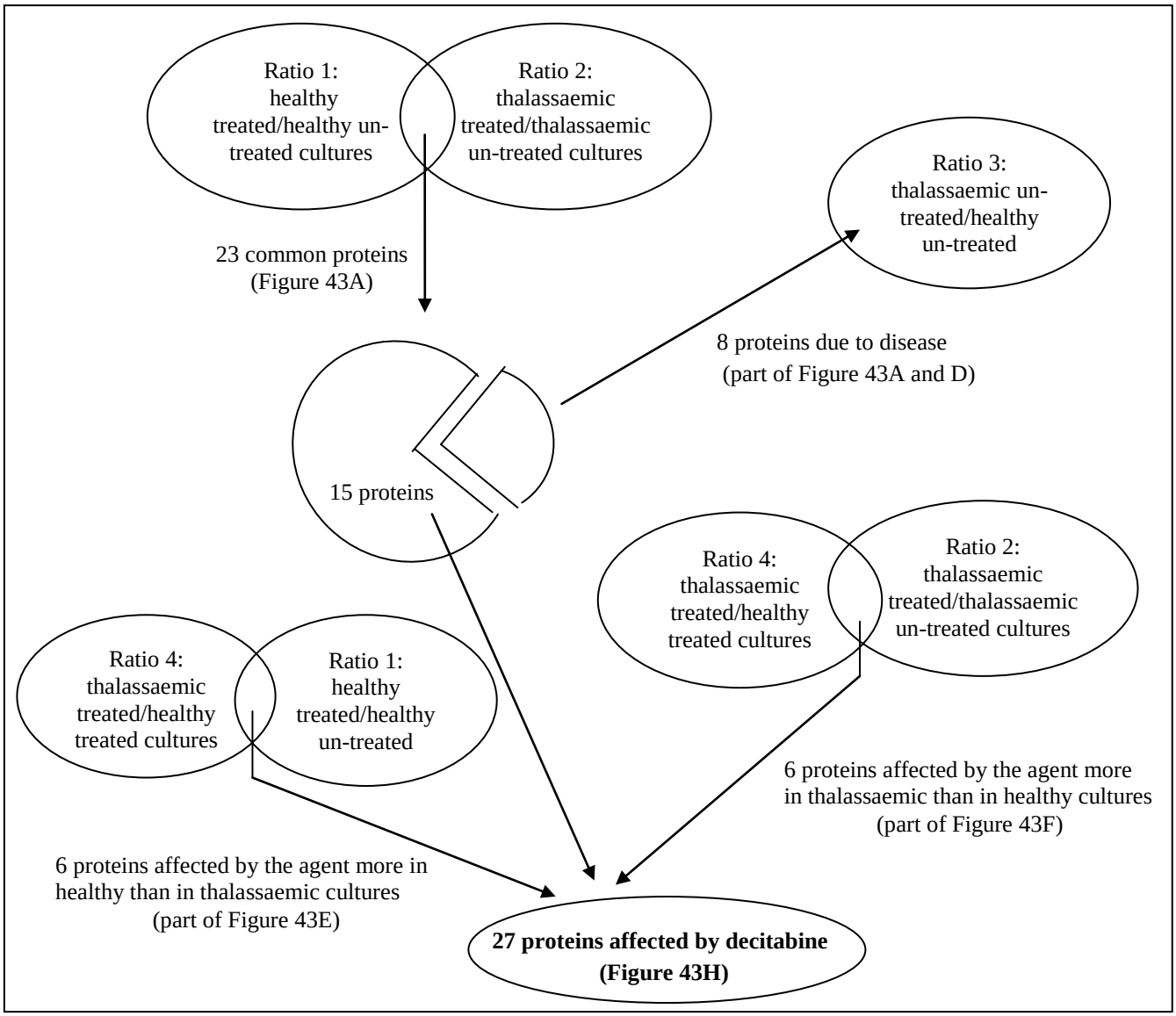


Figure 44, Schematic diagram of the process followed to identify the proteins that are differentially expressed due to decitabine

Table 22, The 27 differentially expressed proteins identified to be potentially involved with the action of decitabine based on the comparative analysis of the differentially expressed protein of the four ratios. The process followed that led to the identification of these 27 proteins is outlined in Figure 44.

Protein ID	***Protein name***	***Function***	***Protein name***	***Ratio 1: healthy treated/healthy un-treated***	***Ratio 2: thal treated/thal un-treated***	***Ratio 4: Thal treated/healthy treated***
Q14738	Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit delta	protein binding in MyD88 dependent toll-like receptor signalling pathway	PPP2R5D	↑	↓	
P51784	Ubiquitin carboxyl-terminal hydrolase 11	inhibits degradation of target proteins by proteosome, regulates pathways leading to NF-κB pathway	USP11	↑	↓	
Q9UDX4	SEC14-like protein 3	transport hydrophobic ligands like tocophenol	SEC14L3	↑	↓	
Q9NP58	ATP-binding cassette sub-family B member 6, mitochondrial	binds haem and porphyrins and mediate with ATP-dependent uptake into mitochondria	ABCB6	↑	↓	
Q8N183	Mimitin, mitochondrial	molecular chaperone for mitochondrial complex I assembly	NDUFAF2	↑	↑	
P40306	Proteasome subunit beta type-10	involved in antigen processing to generate class I binding peptides, involved in ER stress	PSMB10	↑	↓	
P60983	Glia maturation factor beta	stimulate neural regeneration	GMFB	↑	↑	
O95199	RCC1 and BTB domain-containing protein 2	Ran quanyl-nucleotide exchange factor activity	RCBTB2	↑	↓	
Q00169	Phosphatidylinositol transfer protein alpha isoform	catalyzes transfer of PtdIns between membranes	PITPNA	↓	↓	
P61011	Signal recognition particle 54 kDa protein	binds signal sequences of pre-secretory protein	SRP54	↓	↑	
Q9C0D2	Centrosomal protein KIAA1731	centriole formation of stability	KIAA1731	↓	↓	
Q16594	Transcription initiation factor TFIID subunit 9	involved in transcriptional activation, gene regulation associated with apoptosis	TAF9	↓	↓	

Table 22, continues

Q8N960	Centrosomal protein of 120 kDa	involved in microtubule-dependent coupling of the nucleus and centrosome	CEP120	↓	↓	
Q14563	Semaphorin-3A	involved in development of olfactory system	SEMA3A	↓	↓	
Q96HH6	Transmembrane protein 19	Possibly involved in NRF2/KEAP1 pathway	TMEM19	↓	↑	↑
O60888	Protein CutA	form part of complex of membrane proteins attached to acetylcholinesterase	CUTA	↑		↓
P61201	COP9 signalosome complex subunit 2	Regulator of cullin RING ubiquitin ligases, involved in phosphorylation of p53/TP53, c-jun/Jun, IkappaBalpha/NFKB1A	COPS2	↑		↓
O75380	NADH dehydrogenase (ubiquinone) iron-sulfur protein 6, mitochondrial	sensory subunit of mitochondrial membrane respiratory chain NADH dehydrogenase (Complex 1)	NDUFS6	↓		↑
Q96KS0	Egl nine homolog 2	Cellular oxygen sensor that catalyses under normoxic conditions the post-translational formation of HIF alpha proteins	EGLN2	↓		↑
P23786	Carnitine O-palmitoyltransferase 2, mitochondrial	catalytic activity	CPT2	↓		↑
O15111	Inhibitor of nuclear factor kappa-B kinase subunit alpha	Phosphorylates inhibitors of NF-κB – allows ubiquitination of inhibitors of NF-κB and translocation of NF-κB in nucleus for gene activation	CHUK	↓		↑
Q9Y6G3	39S ribosomal protein L42, mitochondrial	poly(A) RNA binding	MRPL42		↑	↑
Q96IZ7	Serine/Arginine-related protein 53	plays a role in pre-mRNA splicing	RSRC1		↑	↑
P04004	Vitronectin	cell adhesion and spreading factor found in serum and tissues	VNT		↑	↑
Q7LDG7	RAS guanyl-releasing protein 2	functions as calcium ad DAG regulated nucleotide exchange factor activating Rap	RASGRP2		↑	↑
P16989	Y-box-binding protein 3	binds to GM-CSF promoter, plays a role in translation repression	YBX3		↓	↓
Q92609	TBC1 domain family member 5	act as GTPase-activation proteins for Rab family proteins	TBC1D5		↓	↓

Similarly, 6 proteins that are commonly differentially expressed in healthy cultures due to the presence of decitabine (healthy treated/healthy un-treated, ratio 1) and in treated samples of thalassaemic over healthy donors (ratio 4) can also be considered as proteins that are being affected by the agent (Figure 44). Addition of the above 12 proteins, to the 15 common proteins that are differentially expressed in thalassaemic and healthy cultures due to decitabine, results in 27 proteins (Figure 43H, gray area, and Table 22) that are considered as the proteins associated with decitabine.

Functional analysis of the 27 proteins (Figure 45) by GeneCodis demonstrated that 16% of the proteins are involved in transport processes. Other biological processes associated with these proteins include negative regulation of apoptosis (6%), protein binding (3%), positive regulation of cell growth (3%), alternative splicing (3%) and the I-kappaB kinase/NF-kappaB signalling pathway (3%) among others.

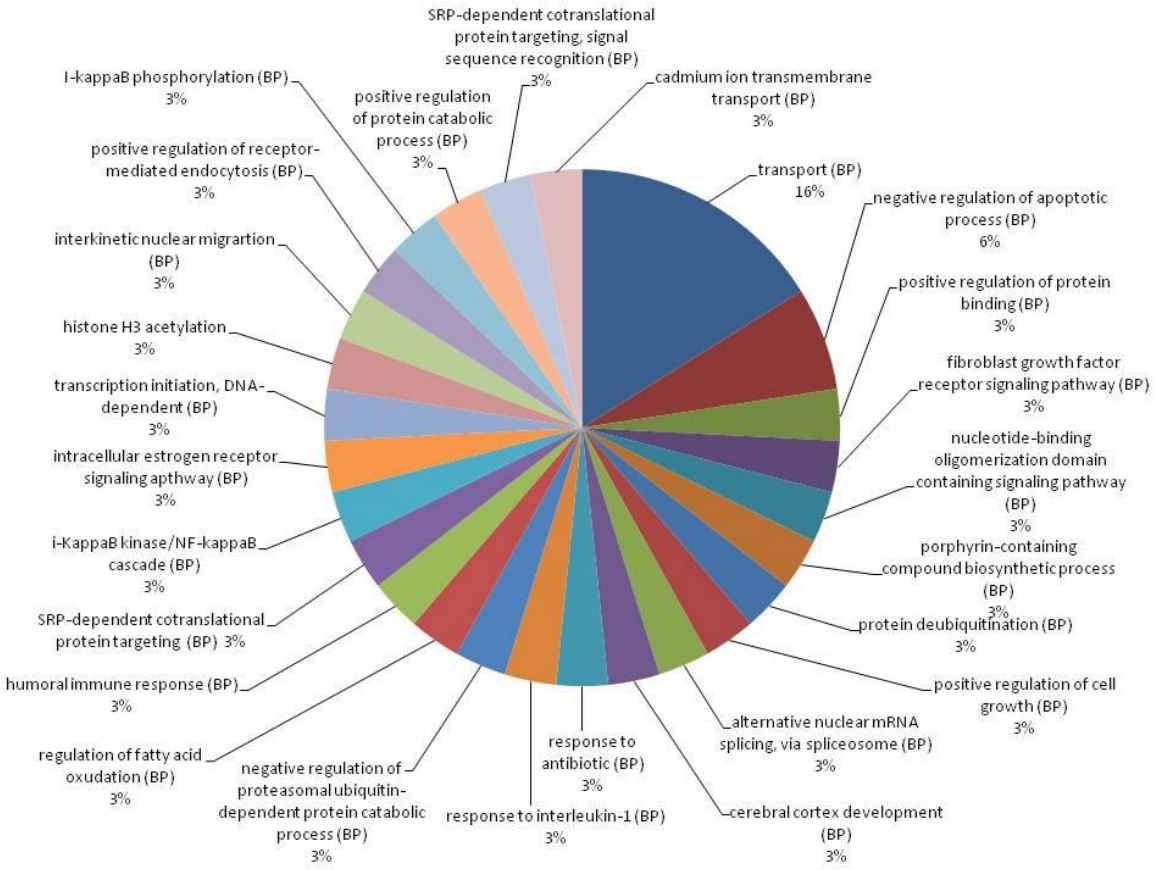


Figure 45, Functional analysis of the 27 proteins whose expression is affected by decitabine. The analysis was performed using Genecodis which categorises proteins according to the biological processes they are involved in.

Although functional annotation using the above bioinformatic tools is useful, their allocation to the different groups depends on the algorithm the particular software is using. We therefore looked into the function of each of the 27 proteins. Based on the function of the 27 proteins (Figure 46), it can be suggested that decitabine promotes transcriptional termination and post-transcriptional processes as can be observed by the up-regulation of SRrp53, a serine/arginine related protein 53 involved in constitutive and alternative splicing (Cazalla *et al.*, 2005), and down-regulation of TAF9, a histone-like TBP-associated factor essential in TFIID mediated transcription (Chen and Manley, 2003). In addition, decitabine might reduce protein ubiquitination and apoptosis through the regulation of the NF-κB signalling pathway supported by the involvement of 3 (COSP2, CHUK and USP11) of the 27 proteins in this pathway (Figure 46). Furthermore, proteins such as EGLN2, a prolyl-4-hydroxylase redox sensor involved in the stabilization of HIF; NDUFAF2, a molecular chaperone for mitochondrial complex I assembly and PSMB10, an IFN-γ mediated proteosome subunit with ATP-dependent proteolytic activity (Foss *et al.*, 1998, Racape *et al.*, 2011) were differentially expressed. These proteins are involved in oxidative and ER stresses possibly leading to activation of signal transduction pathways (Figure 46).

Apart from the NF-κB pathway, decitabine may be involved in the MyD88 dependent toll-like receptor signalling pathway (PPP2R5D) and Ras signalling pathway (RASGRP2) (Figure 46). Activation of these pathways could be either due to demethylation and transcriptional activation of random genes throughout the genome by decitabine or through activation of specific pathways related to oxidative stress such as the HIF signalling pathway.

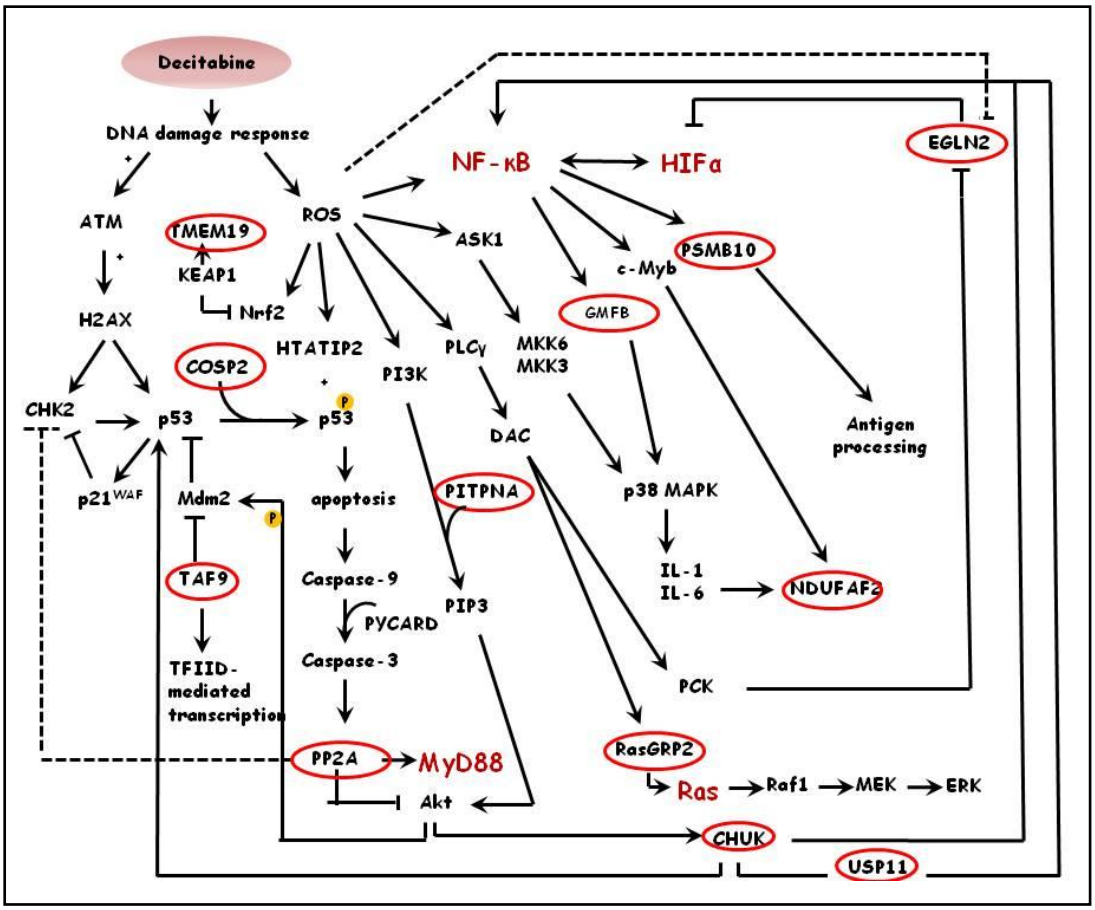


Figure 46, Schematic diagram demonstrating the various pathways potentially involved with the action of decitabine. Differentially expressed proteins (highlighted with red circles) associated with the action of decitabine are involved in the NF-κB, HIF, Ras and MyD88 pathways.

3.6.3. Discussion

To delineate the molecular mechanism of action of decitabine responsible for reactivation of foetal haemoglobin, we employed a quantitative proteomic iTRAQ approach where the identity and relative abundance of each protein in the experimental primary human erythroid cultures could be determined simultaneously.

The major advantage of the iTRAQ technique is its applicability to primary samples such as human tissues. In addition, its ability to analyse multiple samples simultaneously facilitates time course studies or allows analysis of larger number of samples simultaneously, saving sample preparation and analysis time while improving the experimental design (Unwin *et al*., 2005). However, current data analysis methods for iTRAQ fail to report reliable relative protein abundance estimates and suffer with problems of precision and accuracy. Precision is affected by random errors, non-

reproducible and unpredictable fluctuations around the true value, while accuracy is compromised by systemic biases arising from inconsistencies in the iTRAQ labelling efficiency and protein digestion.

The logarithmic transformation has been previously suggested as a method to address the heterogeneity of variance for iTRAQ data (Boehm *et al.*, 2007). For this reason, the abundance ratios of all of the 2188 proteins identified in the current study were converted into their $\log_2$ values. However, attempts to normalise these biases led to compression of ratios towards 1 which lead to underestimation of the ratios as previously shown (Bantscheff *et al.*, 2008, Ow *et al.*, 2009). Bantscheff *et al.* (2008) showed that ratio compression is due to contamination during ion selection, which occurs consistently within an experiment. However, Karp *et al.* (2010) demonstrated that like any other normalization method, logarithm transformation does not sufficiently stabilise the variance.

Despite the above limitations, normalization using the logarithmic transformation allowed us to filter the 2188 identified proteins based on their p-value into 105 differentially expressed proteins (p-value<0.05) in healthy cultures and 110 in thalassaemic cultures in the presence of decitabine. Functional annotation analysis of the differentially expressed proteins demonstrated that decitabine commonly affects proteins that are involved in protein binding, chromatin organization and regulation of transcription in both healthy and thalassaemic cultures. In addition, differentially expressed proteins in both healthy and thalassaemic primary human erythroid cultures treated with decitabine are involved in regulation of the NF-κB pathway.

In order to understand and interpret these data and to generate testable hypotheses on the response of the proteome to decitabine, the list has to be further classified and filtered. 23 proteins were commonly differentially expressed in both healthy and thalassaemic cultures in the presence of decitabine. Filtering out the proteins attributed to the disease state, as defined by the proteins differentially expressed in thalassaemic but not in healthy cultures in the absence of the agent, reduced the potential candidates to 15 proteins. Proteins that were not differentially expressed in thalassaemic or healthy cultures in the presence of decitabine but were found to be differentially expressed when thalassaemic and healthy treated cultures were compared, were considered as proteins affected by the agent and were added to the list of 15 proteins. Functional annotation of the resulting 27 proteins, proteins considered to be associated with the

action of decitabine, showed that these proteins are involved in transport, protein binding and ubiquitination and regulation of cell growth among others. In addition, functional annotation of the above proteins also demonstrated the involvement of decitabine with the NF-κB pathway. NF-κB is active during the early stages of normal erythroid development (Zhang *et al.*, 1998, Jeong *et al.*, 2011) and is responsible for the suppression of a number of transcription factors critical for normal erythropoiesis including MYB, MYC and NFE2. Recently, resveratrol was shown to inhibit TNFα-mediated NF-κB activation and promote erythropoiesis in primary human erythroid cells (Jeong *et al.*, 2011).

However, functional annotation of differentially expressed protein presents many drawbacks. Most of the annotations are done computationally and only a minority is based on experimental data. In addition, the list of terms used for functional annotation is not yet complete and changes with the continuous addition of new discoveries, making the terms redundant or obsolete (Schmidt *et al.*, 2014). Studies have shown that comparison of the different algorithms used by different softwares showed high discrepancies in the functional grouping of the same proteins (Khatri and Draghici, 2005).

Examining the function of each individual protein suggested that decitabine promotes survival of immature erythroid progenitors in thalassaemic cultures, possibly due to modulation of eythropoiesis (Figure 40). Alternatively, oxidative stress in healthy cultures might lead to activation of signal transduction pathways and subsequent activation of γ-globin expression (Figure 38). This agrees with claims that increasing levels of stress such as in the case of haemolysis and hypoxia, lead to increased HbF contents per F cell due to stimulation of maturation of erythroid progenitors that retain the ability to produce HbF (Desimone *et al.*, 1978, Desimone *et al.*, 1982a). Moreover, the toxic effects of 5-azacytidine were suggested to be responsible for the augmentation of HbF synthesis due to accelerated maturation of early progenitors (Torrealbaderon *et al.*, 1984). In addition, signalling pathways, such as the NF-κB pathway, might be activated as a result of the different responses to decitabine observed in healthy and thalassaemic cultures (Figure 46). The potential involvement of the NF-κB signalling pathway in the molecular mechanism of decitabine responsible for HbF reactivation merits further investigation.

The general perception is that MS-based proteomics have low reliability in the complete coverage of proteins, and complexes involved in the same signalling pathway or belonging to the same functional family is not usually achieved (Schmidt *et al.,* 2014). Despite the high mass accuracy of mass spectrometers, challenges of the proteomic investigation occur at all levels, from sample preparation, raw data integration and database searching to the functional interpretation of large datasets (Tabb *et al.,* 2010).

Reproducibility in protein identification among replicates can vary between 30 and 60% (Liu *et al*., 2004, Tabb *et al*., 2010). In complex peptide mixtures such as cell lysates, the number of ions co-eluting can significantly exceed the number of ions for which tandem mass spectra can be acquired, showing a bias towards high abundance ion signals that correspond to peptides found at high levels (Liu *et al.,* 2004). High abundant proteins are usually identified by multiple peptides while low abundant proteins by one or two. In the current study, around 60% of the proteins in the proteomic experiments performed were identified with at least two peptide matches. This percentage coincides with quantitative iTRAQ analysis of *Escerichia coli* cells (Aggarwal *et al.,* 2005). Although protein identification based on multiple peptide matches improves confidence of the identification and quantitation, more than one-third of the proteins in most iTRAQ studies conducted so far have been identified using only one peptide (Aggarwal *et al.,* 2006).

Despite the ability of iTRAQ to detect low abundance proteins, incomplete MS spectra and the use of overly stringent identification criteria would result in missed identifications (Bell *et al*., 2009). Another commonly encountered issue encountered in iTRAQ is the non-random missingness. Missingness refers to the loss of data when peptides are observed at different intensities in different samples (Luo *et al.,* 2009). The probability that a protein is missing is not random, but depends on the abundance of the protein. Low abundance peptides are more likely to be missed due to the data acquisition of the analysis process (Liu *et al.,* 2004). These might explain the inability of the iTRAQ approach in our study to detect seven of the transcription factor investigated by real-time PCR (Section 3.5.3).

Surprisingly, none of the haemoglobin gamma subunits were identified among the differentially expressed proteins in either the healthy or thalassaemic cultures treated with decitabine. In fact, the expression levels of both haemoglobin gamma subunits in the presence of decitabine remained at similar levels to the un-treated counterparts in

both healthy and thalassaemic cultures. In contrast, HPLC analysis of the same cultures demonstrated an increase of 1.7-fold in the percentage of HbF after treatment with decitabine. The lack of detection of significant changes in haemoglobin gamma subunit levels in our proteomic analysis might be attributed to variations within biological replicates. While averaging of replicates to produce a single measure is acceptable, it leads to loss of information about variability. Averaging of replicates was performed in the current study which might have resulted in loss of information. In addition, both haemoglobin gamma subunits were identified with only a single unique peptide despite the large sequence coverage observed, rendering the haemoglobin gamma subunits as low-abundant proteins. Since peptides from more abundant proteins (haemoglobin beta subunit) are more frequently selected than peptides from low abundant proteins (haemoglobin gamma subunit) by the instrument, this questions the accuracy of the measurement for low abundant proteins. In addition, the limited unique peptide matches for haemoglobin gamma subunits might be attributed to sequence similarities between the haemoglobin beta and gamma subunits. Peptides that originated from the haemoglobin gamma subunit but were homologous to haemoglobin beta sequence might have been wrongly assigned (i.e. haemoglobin beta subunit).

Despite the presence of limitations in the accuracy, precision and reproducibility of proteomic approaches, a global protein profiling approach eliminates the need to study drug-induced response of individual cellular pathways and provides a common platform for the simultaneous detection of thousands of drug-related changes in proteins. This allows the generation of new hypotheses for testing of potential pathways and targets and their potential association with the biological states, in our case treatment with decitabine.

3.7. Lentiviral shRNA-mediated knock-downs of selected proteins

3.7.1. Introduction

Proteomic analysis performed on erythroid cultures identified 27 proteins that were significantly differentially expressed in response to decitabine (Table 22). The aim was to investigate their potential role on the expression of the γ-globin genes and their association with the action of decitabine. Due to time constrains, the role of all 27 proteins could not be investigated. Instead, we targeted 17 proteins (Table 23): 6 proteins (USP11, PSMB10, RCBTB2, TAF9, TMEM19, EGLN2), from the list of 27, were selected with the aim of investigating the role of different biological pathways potentially involved in mediating HbF inducing activity of decitabine. The remaining 11 proteins were selected based on their differential expression in the healthy treated/un-treated and thalassaemic treated/un-treated ratios (ratio 1 & 2, Section 3.6): 5 proteins (CHUK, PYCARD, HTATIP2, HEXIM1, ARHGAP4) were significantly differentially expressed only in healthy cultures (i.e. treated/un-treated, ratio 1), 5 (BAZ1B, CHD5, PPP5C, SMARCD3, TACC3) only in thalassaemic cultures (i.e. treated/un-treated, ratio 2) and 1 protein (S100A8) was expressed at higher levels in healthy treated over thalassaemic treated cultures (ratio 4). In order to investigate the effect of each protein, lentiviral shRNA-mediated knock-down experiments were performed for each protein in primary human erythroid progenitor cells from healthy donors in the presence and absence of decitabine.

3.7.2. The effect of lentiviral shRNA-mediated knock-down of 17 target proteins on γ-globin expression

Five different shRNAs were screened simultaneously for each protein to ensure that at least two of the shRNAs would be successful in knocking-down gene expression. All of the shRNAs were incorporated in the pLKO.1 vector. As a negative control and reference sample, the pLKO.1 vector containing a scrambled insert was used to eliminate any transduction-mediated effects. Primary human erythroid cultures from healthy donors were set up according to the previously defined protocol (Section 3.1). On the fourth day of the EPO-dependent phase II of the cultures, transduction of cultures was performed with shRNA expressing lentivirus vectors.

Table 23, List of differentially expressed proteins that were selected for further investigation of their effect on γ-globin gene expression in the presence and absence of decitabine. Ratios of up- or down-regulation of the proteins are presented along with the function of each protein. The ratios represent the fold change in expression of proteins between different cultures as described in section 3.6.2. The arrows demonstrate whether the protein is up- (↑) or down- (↓) regulated in each ratio.

Protein ID	***Protein Description***	***Name***	***Ratio 1: healthy treated/healthy un-treated***		***Ratio 2: thal treated/ thal un-treated***		***Ratio 3: Thal un-treated/healthy un-treated***		***Ratio 4: thal treated/ healthy treated***		***Function***
P51784	Ubiquitin carboxyl-terminal hydrolase 11	USP11	↑	1.652	↓	0.706					ubiquitin/NF-κB
P40306	Proteasome subunit beta type-10	PSMB10	↑	1.289	↓	0.687					ER stress
O95199	RCC1 and BTB domain-containing protein 2	RCBTB2	↑	1.248	↓	0.682					unknown
Q16594	Transcription initiation factor TFIID subunit 9	TAF9	↓	0.805	↓	0.598					transcription activation/repression
Q96HH6	Transmembrane protein 19	TMEM19	↓	0.544	↑	2.099					NRF2/KEAP1 (?)
Q96KS0	Egl nine homolog 2	EGLN2	↓	0.758	↓	0.905			↑	1.567	activation of NF-κB/oxygen sensor
O15111	Inhibitor of nuclear factor kappa-B kinase subunit alpha	CHUK	↓	0.703					↑	1.588	NF-κB/CREBBP activation
Q9UIG0	Tyrosine-protein kinase BAZ1B	BAZ1B			↓	0.656	↑	1.954			chromatin remodelling/WICH
Q9ULZ3	Apoptosis-associated speck-like protein containing CARD	PYCARD	↑	1.289							apoptosis/inhibit CHUK
Q9BUP3	Oxidoreductase HTATIP2	HTATIP2	↑	1.262							redox sensor linked to transcription/demethylated by Decitabine
O94992	Hemamethylene bis-acetamide-inducible protein 1	HEXIM1	↓	0.666							RNA polymerase II transcription inhibitor
P98171	Rho-GTPase activating protein4	ARHGAP4	↓	0.766							Rho-like GTPase
Q8TD10	Chomodomain-helicase binding protein 5	CHD5			↓	0.545					chromatin remodelling/NURD-like complex
P53041	Serine/threonine-protein phosphatase 5	PPP5C			↑	1.111					inhibits ASK1/MAP3K5/ERK
Q6STE5	SWI/SNF related matrix associated actin dependent regulator of chromatin subfamily D member 3	SMARCD3			↑	1.096					ATP dependent nucleosome remodelling
Q9Y6A5	Transforming acidic coiled-coil containing protein 3	TACC3			↑	1.331					FOG1interacting protein
P05109	Protein S100-A8	S100A8							↓	0.514	calprotein/TLR4 binding/scavening oxidant

In order to allow recovery of the cultures and maximum knock-down of the proteins, decitabine was added on the sixth day of EPO-dependent phase II, i.e. two days after transduction of primary human erythroid cultures, at a concentration of 300nM. HPLC analysis confirmed that both of the primary human erythroid cultures set up from healthy donors for lentiviral shRNA-mediated knock-downs, were good responders to decitabine (Table 24). The HbF percentage doubled after decitabine treatment in both cultures.

Table 24, Levels of HbF induction in primary human erythroid cultures treated with 300nM decitabine. The HbF percentage of the primary human erythroid cultures used for lentiviral shRNA-mediated knock-downs was determined before and after the addition of the decitabine by HPLC. The table also demonstrates the percentage increase in HbF after treatment.

Healthy donors	***HbF levels (%)***		***% increase in HbF***
	un-treated	***300nM Dec***	
1	11.14	26.38	136.80
2	3.32	20.28	510.80

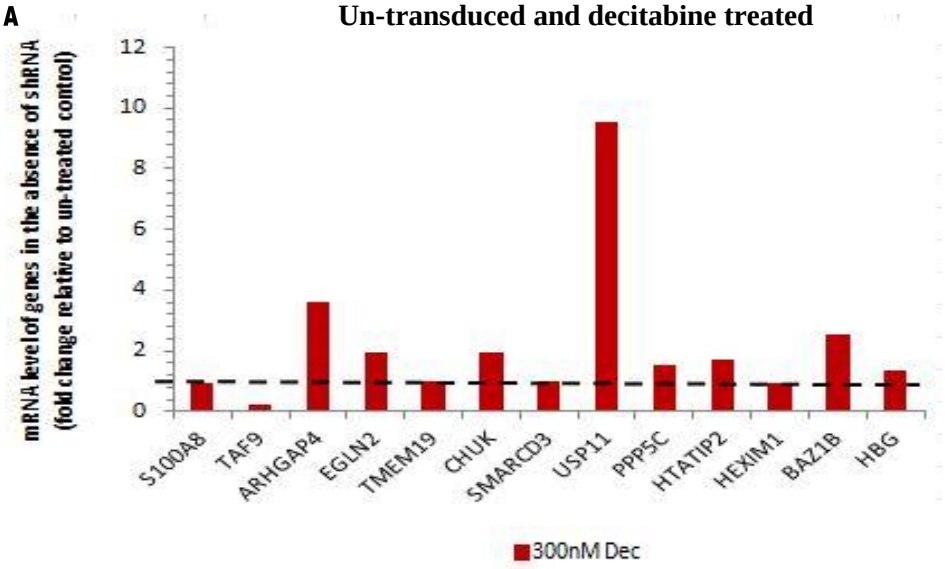
Before investigating the effect of knocking-down each of the above 17 proteins, the effectiveness of each shRNA in reducing the mRNA levels of each corresponding gene was investigated by real-time PCR. Knock-down of each gene was considered successful when more than one shRNA clone was capable of reducing the mRNA levels of that gene. This will allow the elimination of false positive results. A threshold of 20% was used as an effective reduction level in the gene expression for an shRNA. The number of effective shRNAs for each protein is listed in Figure 47.

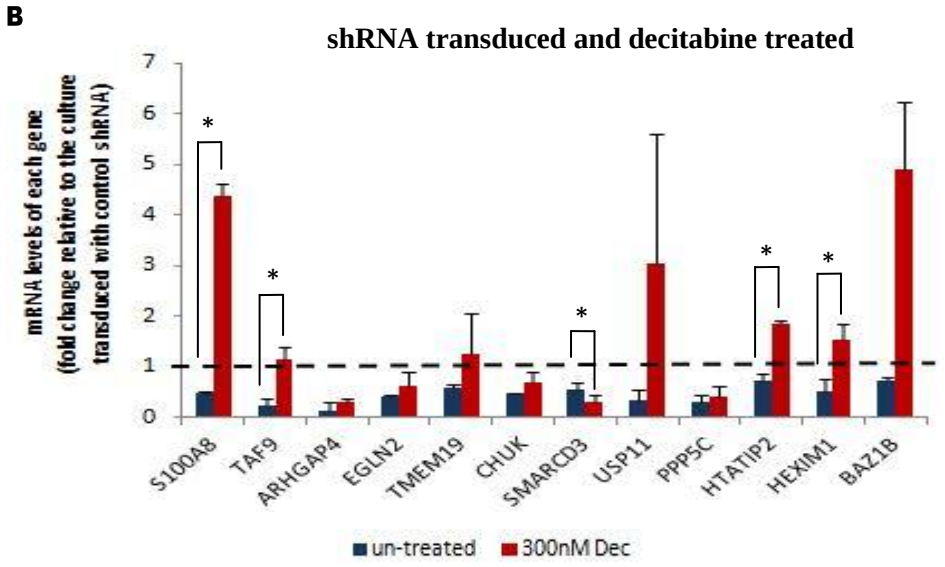
The shRNAs destined for four genes were not considered successful in knocking-down their respective gene expression. In the case of *TACC3* and *RCBTB2* genes, in fact, there was an increase rather than a decrease in the expression levels of the genes relative to the pLKO.1 scramble control (ratio of 1) as shown by qRT-PCR (Figure 47). In the case of *PSMB10* and *PYCARD* genes, shRNAs were able to decrease gene expression by only 5 % and were therefore not considered successful.

Protein name	*shRNA clone*					# of effective shRNA clones
	1	*2*	*3*	*4*	*5*	
S100A8	51.17983758	-12.26222	-240.55422	50.25217		2
PSMB10	-162.3595853	-92.422566	6.4945734	-77.027869		0
TAF9	87.03744514	59.35947	83.47049	50.94225	73.3780252	5
ARHGAP4	70.27614792	23.428523	100	58.508794	86.26573129	5
EGLN2	57.1202916	63.123682	-76.815715	55.492188	19.99716665	4
TMEM19	-41.17459573	-46.843001	46.006019	49.367733	32.66037931	3
CHUK	54.54843108	52.3871	-11.5416	29.57864	-1.4123251	2
SMARCD3	35.35812279	51.269971	5.8886259	46.081379	-1.118817667	3
USP11	45.6168358	64.871132	90.654669	91.98975	73.73688401	5
TACC3	-240.4582752	-75.18503	-118.90266	-10.036793	-150.9557496	0
PPP5C	63.77933347	63.61228	89.14636	60.94523	64.7460211	5
RCBTB2	-180.1244673	-858.72131	-316.22828	-98.571696	-473.7578551	0
HTATIP2	36.90948642	16.428149	26.949261	21.102828	4.232137958	2
HEXIM1	31.2566054	65.192382	37.242143	18.56505	-14.31201112	3
PYCARD	5.273006581	-44.743895	-6.4034921	5.7689124	-3.486422685	0
BAZ1B	21.65011838	-68.9517	-9.30194	36.12304	-43.491323	2

Figure 47, Heat map showing the reduction in gene expression of the 16 proteins mediated by each of their 5 shRNAs. Each row of the heat map represents the 5 shRNAs used for knock-down of each protein and the percentage reduction in their mRNA. The scale of reduction is indicated from 0 for no reduction in mRNA levels, to 100 for complete loss of mRNA production. The last column to the right indicates the number of successful shRNAs for each protein; a 20% reduction in mRNA levels was used as the threshold for successful protein knock-down. The data represent the average of triplicates of one biological replicate for each shRNA in primary human erythroid cultures from healthy donors. Note: Only 16 of the 17 genes are shown, since it was not possible to examine the expression levels of CHD5 due to failure of two sets of primers to detect any products by quantitative real-time PCR.

For the remaining 12 proteins (S1000A8, TAF9, ARHGAP4, EGLN2, TMEM19, CHUK, SMARCD3, USP11, PPP5C, HTATIP2, HEXIM1 and BAZ1B) that could be knocked-down, we initially assessed the changes in mRNA levels of the genes under investigation in un-transduced (without knock-down) but decitabine treated primary human erythroid cultures (Figure 48A), followed by the effect of the shRNAs with and without decitabine treatment on the expression of the gene itself (Figure 48B) and on γ-globin expression (Figure 48C and 49). A summary of the results is presented in table 25.


A
Un-transduced and decitabine treated
mRNA level of genes in the absence of shRNA (fold change relative to un-treated control)
12
10
8
6
4
2
0
S100A8
TAF9
ARHGAP4
EGLN2
TMEM19
CHUK
SMARCD3
USP11
PPP5C
HTATIP2
HEXIM1
BAZ1B
HBG
300nM Dec


B
shRNA transduced and decitabine treated
mRNA levels of each gene (fold change relative to the culture transduced with control shRNA)
7
6
5
4
3
2
1
0
*
S100A8
TAF9
ARHGAP4
EGLN2
TMEM19
CHUK
SMARCD3
USP11
PPP5C
HTATIP2
HEXIM1
BAZ1B
un-treated
300nM Dec

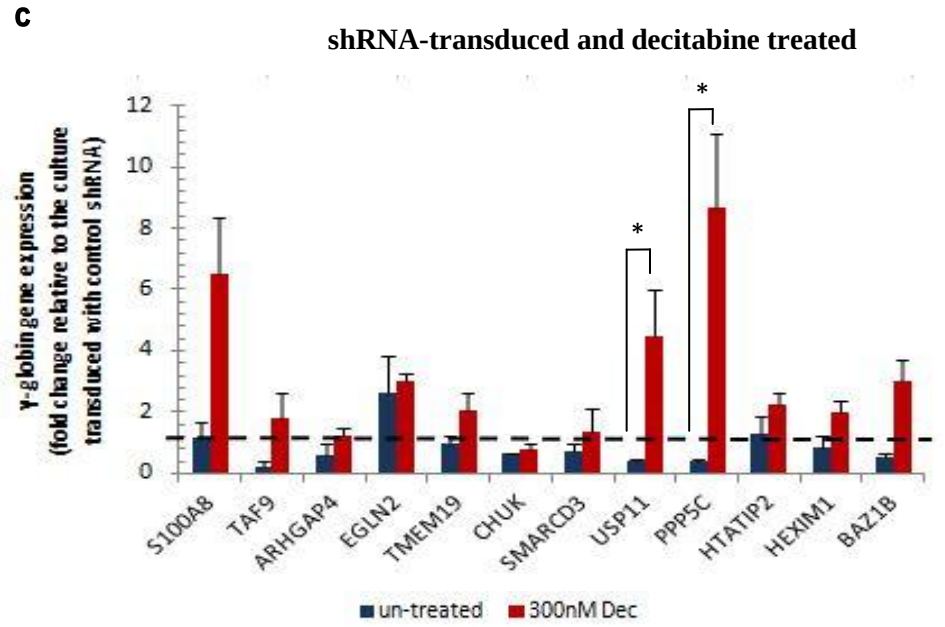

C
shRNA-transduced and decitabine treated
γ-globin gene expression (fold change relative to the culture transduced with control shRNA)
12
10
8
6
4
2
0
*
S100A8
TAF9
ARHGAP4
EGLN2
TMEM19
CHUK
SMARCD3
USP11
PPP5C
HTATIP2
HEXIM1
BAZ1B
un-treated
300nM Dec

Figure 48, mRNA levels of genes under investigation and γ-globin in un-transduced and shRNA-transduced primary human erythroid cultures from healthy donors, in the presence and absence of decitabine. (A) Changes in gene expression levels of the genes under investigation in un-transduced primary human erythroid cultures following treatment with decitabine. Lentiviral shRNA-mediated knock-down of each gene was carried out in primary human erythroid cultures, where the effect on gene expression levels of each gene under investigation (B) and γ-globin (C) were investigated by quantitative real-time PCR. The mRNA levels in A are expressed as fold change relative to the un-transduced cultures (ratio of 1, dotted line) while the mRNA levels in B & C are expressed as fold change relative to the culture transduced with control shRNA (ratio of 1, dotted line). The results represent the average of all effective shRNAs (2-5 clones) corresponding to each protein, that were originated from a single experiment from one biological replicate. Error bars correspond to the standard deviation. * correspond to statistically significant (p-value <0.05) changes in gene expression in the presence of decitabine when compared to the un-treated samples transduced with the corresponding shRNA for each protein according to the paired t-test.

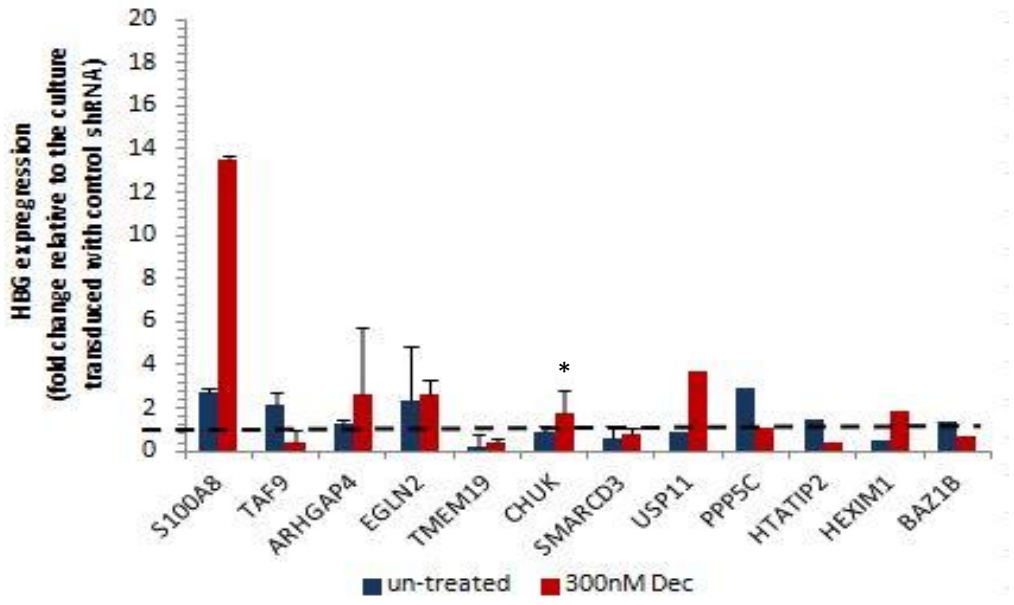


Figure 49, Western blot analysis of HBG expression levels in primary human erythroid cultures from healthy donors transduced with shRNA expressing lentivirus for each of the 12 genes (horizontal axis) under investigation, in the absence (blue bars) and presence (red bars) of decitabine. HBG expression levels are expressed as a fold change relative to the culture transduced with control shRNA (dotted line, ratio of 1). The results represent the average fold change of HBG levels for all the effective shRNAs that correspond to each protein. Error bars correspond to the standard deviation. * corresponds to statistically significant (p-value<0.05) changes in HBG expression according to the paired t-test.

Treatment of un-transduced primary human erythroid cultures with decitabine increased the expression levels of *ARHGAP4*, *EGLN2*, *CHUK*, *USP11*, *PPP5C*, *HTATIP2* and *BAZ1B* along with *HBG*. The expression levels of the remaining genes remained unaffected by decitabine, with the exception of *TAF9*, whose expression decreased after treatment (Figure 48A and Table 25).

Looking at each of the knocked-down genes individually, it was observed that reduced expression of *S100A8* gene did not affect *HBG* expression levels (Figure 48B). Decitabine treatment of primary human erythroid cultures already transduced with the effective shRNA clones, resulted in a significant (p-value<0.05) increase in the expression level of S100A8 itself along with a substantial increase in *HBG* expression (Figure 48B & C). This suggests that decitabine might increase expression of the gene enough to overcome the knock-down effect. However, this was not represented by the response of S100A8 to decitabine in un-transduced samples, which did not change relative to the un-treated control (Figure 48A). Western blot analysis, in contrast to the real-time PCR, showed an increase in HBG expression after reducing the expression levels of *S100A8*, which was enhanced after treatment with decitabine (Figure 49).

Knock-down of *TAF9* reduced *HBG* expression in the absence of decitabine. Following decitabine treatment, there was a significant increase in the mRNA levels of the gene to similar levels as the negative control (Figure 48B) and an increase in *HBG* expression (Figure 48C). Interestingly, decitabine seems to decrease TAF9 expression in the absence of shRNAs (Figure 48A). Western blot analysis showed completely opposite results to the real-time PCR analysis, with an increased HBG expression following knock-down of *TAF9* which decreases after treatment with decitabine (Figure 49).

Decitabine treatment of primary human erythroid cultures prior to knock-down, increased *ARHGAP4* expression by approximately 4-fold above the levels of the un-treated control (Figure 48A). Reduced expression of *ARHGAP4* led to reduced *HBG* mRNA levels in the absence of decitabine (Figure 48C) while maintained HBG expression to similar levels to the negative control (Figure 49). In the presence of decitabine, *ARHGAP4* increased slightly but still remained suppressed (Figure 48B) by the shRNAs while increased γ-globin expression both at the mRNA (Figure 48C) and protein level (Figure 49).

EGLN2 gene expression increased following decitabine treatment in un-transduced primary human erythroid cultures (Figure 48A). Knock-down of *EGLN2* in the absence of decitabine was accompanied by an increase in *HBG* expression (Figure 48C). Following treatment with decitabine, *EGLN2* expression increased slightly but remained suppressed in the presence of the shRNA clones (Figure 48B), while *HBG* mRNA expression increased further (Figure 48C). HBG protein expression was increased following reduction of *EGLN2* and was further increased non-significantly in the presence of decitabine (Figure 49).

TMEM19 and *HEXIM1* expression remained unchanged after decitabine treatment in the absence of shRNAs (Figure 48A). Reduced expression of both genes resulted in a small reduction in *HBG* expression in the absence of decitabine. Treatment with the agent increased both the expression of the genes themselves (Figure 48B), significantly in the case of *HEXIM1*, and *HBG* expression (Figure 48C). At the protein level, HBG expression was reduced following knock-down of both genes which was increased above the negative control only in the case of *HEXIM1* following treatment with decitabine (Figure 49).

CHUK expression increased following decitabine treatment in both un-transduced (Figure 48A) and transduced cultures (Figure 48B). However, the increase in expression in the presence of shRNAs was only minimal (Figure 48B). Knock-down of *CHUK* expression was associated with a decrease in γ-globin expression at both the mRNA (Figure 48C) and protein level (Figure 49) in the absence of decitabine. Western blot analysis demonstrated an increase in HBG expression following decitabine treatment in the presence of CHUK knock-down (Figure 49).

SMARCD3 was the only gene that showed further decrease in expression after shRNA transduction and decitabine treatment (Figure 48B), a decrease that was statistically significant ($p\text{-value}<0.05$). The decreased *SMARCD3* expression was accompanied by a small increase in *HBG* expression (Figure 48C) which was not substantial at the protein level (Figure 49).

USP11 showed the highest increase in expression following decitabine treatment in un-transduced primary human erythroid cultures (Figure 48A). In the presence of decitabine, *USP11* gene expression also increased above the levels of the negative control despite the successful knock-down of the gene by shRNAs (Figure 48B). The

increased expression of *USP11* following decitabine treatment, was accompanied by a significant (p-value<0.05) increase in γ-globin expression (Figure 48C). Up-regulation of HBG expression in the presence of decitabine was also observed at the protein level (Figure 49). This was confirmed by the proteomic analysis which showed an increased expression of USP11 in healthy cultures following decitabine treatment. Interestingly, USP11 was significantly decreased in thalassaemic cultures treated with decitabine as shown by the proteomic analysis (Table 23).

Knock-down of *PPP5C* expression reduced *HBG* expression in the absence of decitabine. After treatment with decitabine, *PPP5C* gene expression remained unchanged (Figure 48B). In contrast, there was a large and significant (p-value<0.05) up-regulation of *HBG* expression following decitabine treatment (Figure 48C). However, this was not represented at the protein level (Figure 49), where in fact a decrease was observed in HBG expression following treatment with decitabine.

HTATIP2 and *BAZ1B* expression increased after treatment with decitabine in un-transduced primary human erythroid cultures (Figure 48A). Knock-down of both genes was accompanied by reduced expression of HBG expression in the absence of decitabine (Figure 48C). Treatment with decitabine not only increased the expression of *HBG* (Figure 48C) but also increased both gene expression levels (Figure 48B). The increase in *HTATIP2* expression was significant following treatment with decitabine. Western blot analysis showed opposite results to the real-time PCR experiments for both genes, with decitabine treatment resulting in decreased HBG expression (Figure 49).

Despite the lack of statistical significance in the changes of HBG expression by decitabine after transduction of primary human erythroid cultures with shRNA against *ARHGAP4*, *ELGN2*, *HEXIM1, S100A8* and *SMARCD3*, western blot analysis has shown some interesting results (Figure 50 and 51).

Down-regulation of *ARHGAP4* with shRNA 47192 (clone 5) resulted in the up-regulation of HBG expression in un-treated cultures. Treatment with decitabine showed an increase in HBG expression with shRNA 47188 (clone 1) and shRNA 47190 (clone 3) (Figure 50A) while maintaining reduced expression of *ARHGAP4* gene expression (Figure 49B). The high HBG expression in the presence of shRNA 47192 (clone 5) remained at similar levels in the presence of decitabine. All of the shRNAs for this gene

are targeting within the coding sequence of the gene. Proteomic analysis showed a decrease in ARHGAP4 protein expression in healthy cultures in the presence of decitabine (Table 23) suggesting an inverse association between *ARHGAP4* gene and γ-globin expression. However, this is in contrast to the increase observed in *ARGHAP4* mRNA levels after treatment with decitabine in un-transduced cultures (Figure 48A).

Down-regulation of *EGLN2* gene expression by shRNA 22324 (clone 1), 22327 (clone 4) and 22328 (clone 5), resulted in up-regulation of HBG expression in the absence of decitabine (Figure 50B) suggesting an inverse relation of the protein with γ-globin expression. Once treated with decitabine, HBG expression was increased further for shRNA 22324 (clone 1) as well as for shRNA 22325 (clone 2) but was reduced for shRNA 22328 (clone 5). shRNA 22324 (clone 1) targets the 3' UTR of the gene while shRNA 22328 is targeting the coding sequence of the gene. The reduced expression of HBG might be due to the removal of inhibition by decitabine through induction of transcription of the gene. A small increase in *EGLN2* expression was observed at the mRNA level in the presence of decitabine (Figure 48A). However, proteomic analysis showed that EGLN2 protein was down-regulated in both healthy and thalassaemic cultures treated with decitabine (Table 23).

Down-regulation of *HEMIX1* gene expression reduced HBG expression with shRNA 74173 (clone 1) and shRNA 74174 (clone 2) in the absence of decitabine (Figure 50C). HBG expression increased after treatment with decitabine in the presence of both shRNAs. Since there is a significant increase in *HEXIM* mRNA level with both shRNA after addition of decitabine (Figure 48B), the increase in γ-globin expression may be attributed to the increase in *HEXIM1* (Figure 48B). In contrast, proteomic analysis showed reduced levels of HEXIM1 in healthy cultures when treated with decitabine (Table 23).

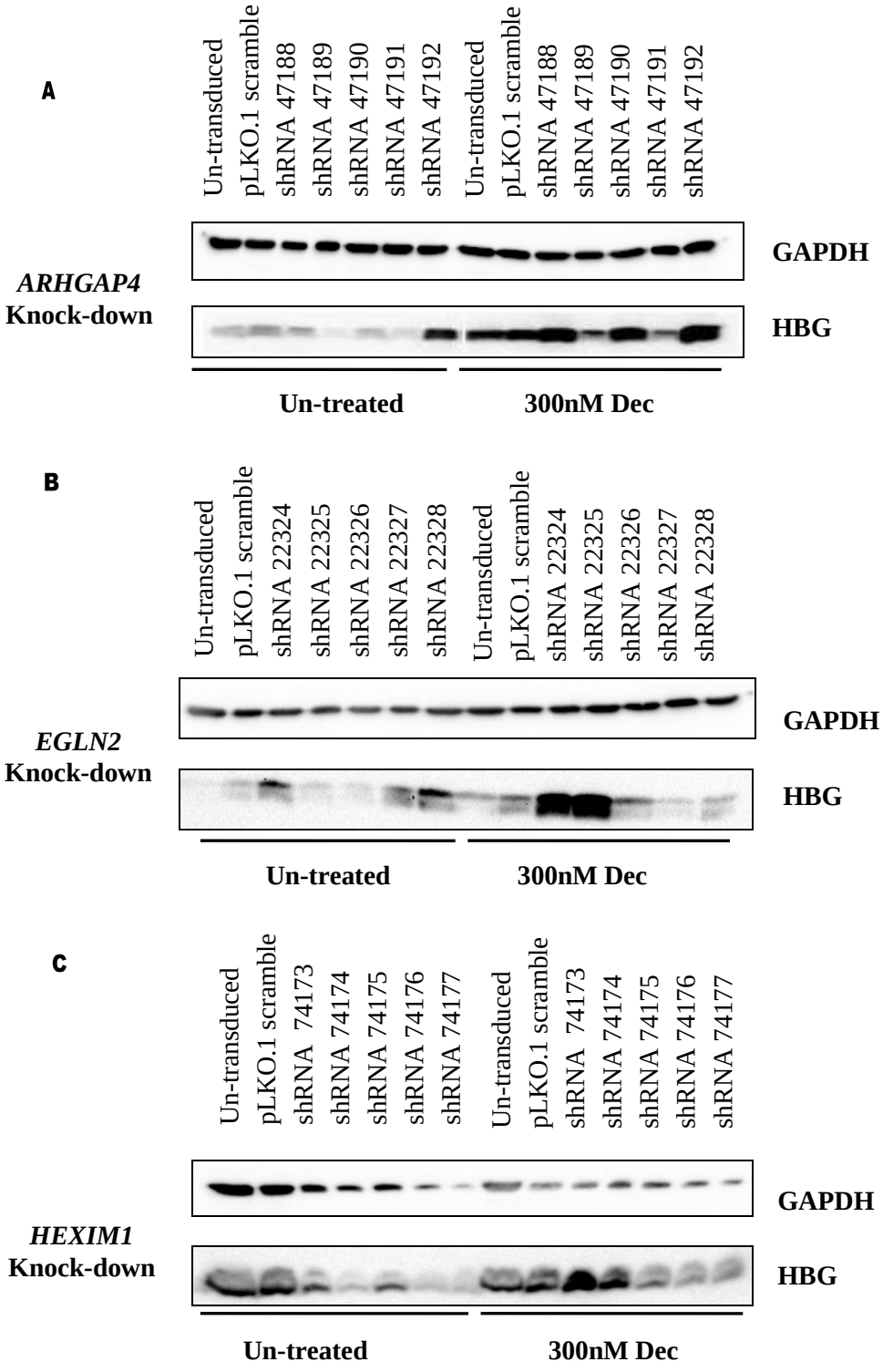


Figure 50, Western blot analysis investigating the HBG levels of expression in primary human erythroid cultures from a healthy donor transduced with shRNAs for *ARHGAP4* (A), *ELGN2* (B) and *HEXIM2* (C) in the absence and presence of 300nM decitabine. HBG expression was normalised to the endogenous GAPDH expression.

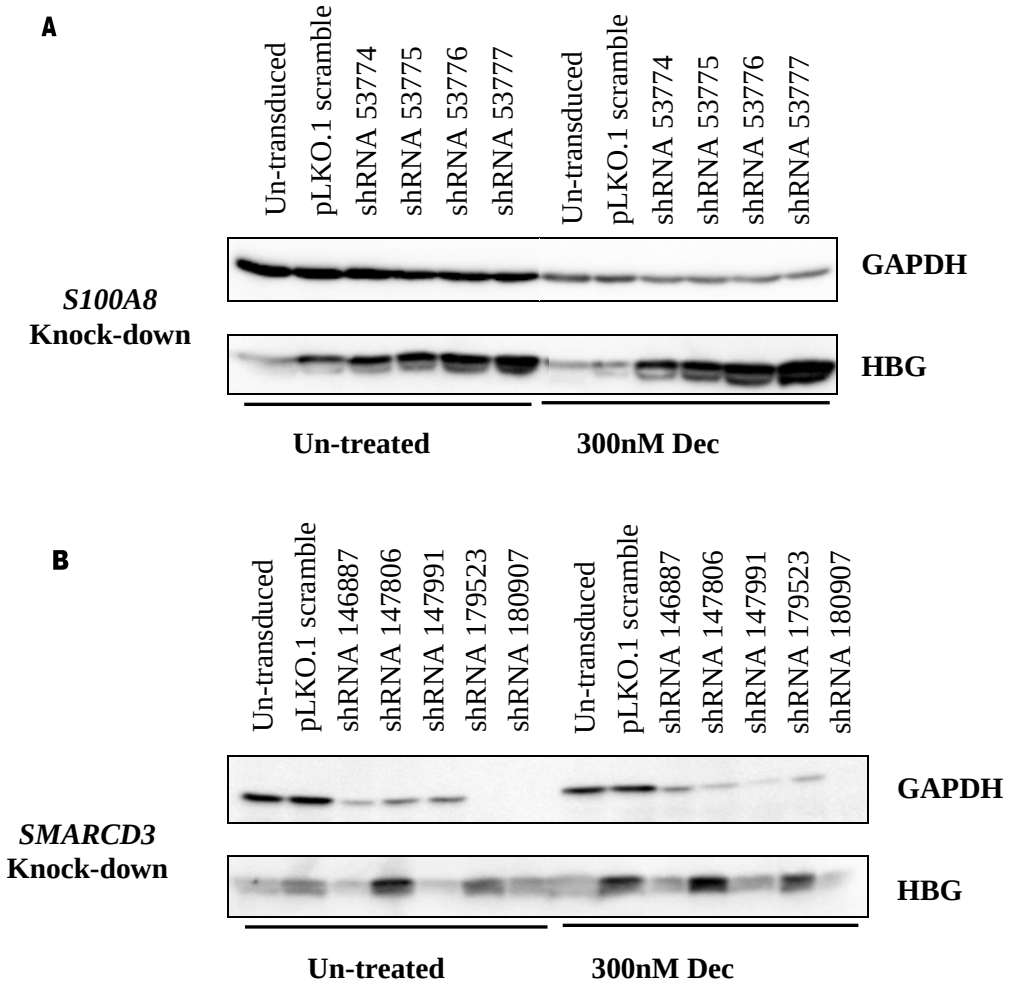


Figure 51, Western blot analysis investigating the HBG levels of expression in primary human erythroid cultures from a healthy donor transduced with shRNAs for *S100A8* (A) and *SMARCD3* (B) in the absence and presence of 300nM decitabine. HBG expression was normalised to the endogenous GAPDH expression.

Down-regulation of *S100A8* expression with shRNAs 53774 (clone 1) and 53777 (clone 4) (Figure 51A), resulted in a small increase in HBG expression relative to pLKO.1 scramble in un-treated cultures, which was further enhanced in the presence of decitabine. Although not statistically significant, the increase in γ-globin expression was observed at both the mRNA (Figure 48C) and protein level (Figure 49) following addition of decitabine. *S100A8* gene expression levels were also increased significantly in the presence of decitabine (Figure 48B) suggesting a loss of repression of the *S100A8* gene in the presence of decitabine. As shown by the proteomic analysis (Section 3.6), S100A8 protein was expressed more in healthy rather than in thalassaemic cultures treated with decitabine (Table 23), and was slightly increased in healthy cultures in the presence of decitabine.

Moreover, reduction of *SMARCD3* expression by shRNA 147806 (clone 2) and 179523 (clone 4) (Figure 51B) resulted in reduced or similar levels of HBG with pLKO.1 scramble suggesting no major effect of this gene on γ-globin expression. However, treatment with decitabine resulted in significantly lower expression levels of *SMARCD3* (Figure 48B) while maintaining or increasing slightly HBG expression (Figure 51B). This suggests that SMARCD3 might be an inhibitor of γ-globin expression so that when decitabine reduces its expression the inhibition is reversed. The small increase in γ-globin expression might suggest that SMARCD3 might act through interaction with other factors and reversion of inhibition might not be high enough to promote high γ-globin induction. In contrast to the real-time PCR, proteomic analysis showed an increased (although marginal) in SMARCD3 expression in thalassaemic cultures when treated with decitabine (Table 23) while remained unchanged in healthy cultures.

Table 25, Summary of the effect of knock-down of the 12 genes under investigation on the gene itself and γ-globin expression in primary human erythroid cultures from healthy donors. Initially, the changes in mRNA levels of the genes under investigation were assessed in un-transduced (without knock-down) but decitabine treated primary human erythroid cultures, followed by the effect of shRNAs with and without decitabine treatment on the expression of the gene itself and on γ-globin expression. The table represents the fold changes in the mRNA and protein levels in the presence of decitabine relative to the un-treated control. * demonstrates the statistically significant changes (p-value<0.05) according to the paired t-test.

	Gene expression			***γ-globin gene expression***	
	mRNA (un-transduced treated/ un-treated)	***mRNA (transduced treated/ un-treated)***	***proteomic (healthy treated/ un-treated)***	***mRNA (treated/ un-treated)***	***HBG (treated/ un-treated)***
S100A8	0.91	8.87*	1.18	5.72	4.88
TAF9	0.25	4.97*	0.81	7.63	0.21
ARHGAP4	3.60	2.05	0.77	2.03	2.00
EGLN2	1.95	1.51	0.75	1.16	1.14
TMEM19	1.02	2.20	0.54	2.17	1.96
CHUK	1.95	1.49	0.70	1.17	1.97*
SMARCD3	0.99	0.53*	1.07	1.83	1.32
USP11	9.52	9.23	1.65	11.51*	3.96
PPP5C	1.54	1.27	1.01	21.22*	0.38
HTATIP2	1.72	2.54*	1.26	1.75	0.28
HEXIM1	0.94	2.99*	0.67	2.38	3.53
BAZ1B	2.57	6.89	1.16	5.30	0.54

3.7.3. Discussion

Based on the proteomic analysis, 17 proteins were selected for further investigation of their effect on γ-globin expression in primary human erythroid cultures using shRNA technology. Among the 17 genes, the expression of *TACC3, PSMB10, RCBTB2* and *PYCARD* could not be repressed efficiently with any of the shRNA tested and were therefore excluded from the analysis. shRNAs within plasmids require nuclear penetration and translational processing to produce the hairpin shRNA for Dicer processing (Siolas *et al.*, 2005). The lack of effective knock-down might be due to ineffective penetration of the plasmid into the cells and ineffective expression of the plasmids or due to the presence of a regulatory feedback for the genes that leads to increased mRNA levels even after transduction. In addition, quantitation of the expression levels of gene *CHD5* was not possible. CHD5 was therefore excluded from the study too due to lack of confirmation of the shRNA's effectiveness.

Successful repression of gene expression for the remaining twelve genes was shown by quantitative real-time PCR. In the absence of decitabine, reduced expression of the genes under investigation was accompanied by either a reduction or no change in γ-globin gene expression in primary human erythroid cultures. The only exception was *EGLN2*, whose reduction resulted in an up-regulation of γ-globin gene expression at both the mRNA and protein levels in the absence of decitabine. Treatment with decitabine resulted in a statistically significant up-regulation of *S100A8, TAF9, HTATIP2* and *HEXIM1* gene expression and a statistically significant down-regulation of *SMARCD3* gene expression in transduced primary human erythroid cultures (Table 25). Moreover treatment with decitabine in the presence of the shRNAs resulted in a significant increase in γ-globin gene expression in *USP11* and *PPP5C* knock-down cultures. This effect was only confirmed at the protein level for *USP11* (Table 25).

USP11 expression increased profoundly after decitabine treatment in both un-transduced cultures and in the presence of the shRNA (Table 25). This was confirmed also in the proteomic analysis where USP11 protein was significantly increased in primary human erythroid cultures from healthy donors treated with decitabine. USP11, a de-ubiquitinating enzyme, was recently shown to inhibit TNFα-induced activation of NF-κB pathway and to regulate IKKα by a de-ubiquitination-independent mechanism (Yamaguchi *et al.*, 2007a) (Figure 52). *CHUK*, known as the inhibitor of nuclear factor

kappa-B kinase subunit alpha (IKKα), was the only gene whose down-regulation resulted in a statistically significant, yet small, increase in HBG expression in the presence of decitabine (Table 25). In contrast to the real-time PCR results, proteomic analysis demonstrated a decrease in CHUK expression in healthy cultures treated with decitabine. Apart from its involvement in NF-κB pathway, IKKα was recently shown to regulate the ROS-induced activation of the p53 tumour suppressor following activation by the protein kinase Cδ (Yamaguchi *et al.*, 2007b) (Figure 52).

Western blot analysis suggests that *ARHGAP4*, *EGLN2, HEXIM1, S100A8* and *SMARCD3* genes might be involved in the mechanisms of action of decitabine in reactivating γ-globin expression.

ARHGAP4 (p115 RhoGAP) encodes for RhoGTPase activating protein 4 that prevents the association of MEKK1 with components of the JNK MAP kinase (Christerson *et al.*, 2002) and promotes PRL-1 mediated ERK1/2 and RhoA activation (Bai *et al.*, 2011) (Figure 53). ARHGAP4 is a potent inhibitor of cell and axon motility (Vogt *et al.*, 2007) that is known to inhibit the formation of stress fibres. In addition, ARHGAP4 has been found to be highly expressed in cells of haematopoietic origin including the K562 cell line (Tribioli *et al.*, 1996). *ARHGAP4* gene was one of the genes found to be induced after hydroxyurea administration leading to signal transduction and transcriptional activation of down-steam targets (Costa *et al.*, 2007). Reduced expression of *ARHGAP4* leads to activation of MEKK1 which in turn activates transcriptional regulator AP-1 and NF-κB, MAP kinase cascades, the c-Jun-N-terminal kinase/stress activated protein kinases and to a lesser extent ERK1/2. In the current study, decitabine treatment increased the mRNA levels of *ARHGAP4* in un-transduced cultures but down-regulated ARGHAP4 protein in proteomic analysis of healthy cultures treated with decitabine. Knock-down of *ARHGAP4* gene expression resulted in induction of HBG expression in the presence of the agent suggesting an inverse relation between the gene and γ-globin expression.

EGLN2, also known as prolyl-4-hydroxylase (PHD1), is responsible for post-translational modification of prolines on specific target proteins (Moser *et al.*, 2013). Under normoxic conditions HIFa is hydroxylated by PHD1-3, which is then recognised by the von Hippel-Lindau protein E3 ubiquitin ligase complex and targeted for proteosomal degradation. Hypoxia reduces the propyl-hydroxylation by PHDs resulting

in stabilization of the HIF complex leading to transcriptional activation of downstream genes including *EPO* (Figure 52). While inhibition of EGLN2 alone did not cause significant effects on EPO induction, knock-down of EGLN1 and ELGN2 have a synergistic effect on EPO induction in Hep3B cells (Fisher *et al.*, 2009) and in mice (Minamishima and Kaelin, 2010). Moreover, Moser *et al.* (2013) have shown that EGLN2 is required for centrosome duplication and maturation through hydroxylation of Cep192 leading to regulation of cell cycle progression in the presence of hypoxia. Over-expression of EGLN2 inhibited NF-κB activity through negative regulation of IKKβ (Cummins *et al.*, 2006)(Figure 53), suggesting an inhibitory role of EGLN2 on NF-κB leading to cell cycle arrest through NF-κB-mediated regulation of cyclin D1 (Xie *et al.*, 2014b) (Figure 52). In the current study, treatment of primary human erythroid cultures from healthy and thalassaemic donors with decitabine resulted in down-regulation of the EGLN2 protein while it increased *EGLN2* mRNA levels. Knock-down of EGLN2 in primary human erythroid cultures led to increased HBG expression at the protein and mRNA level (Table 25).

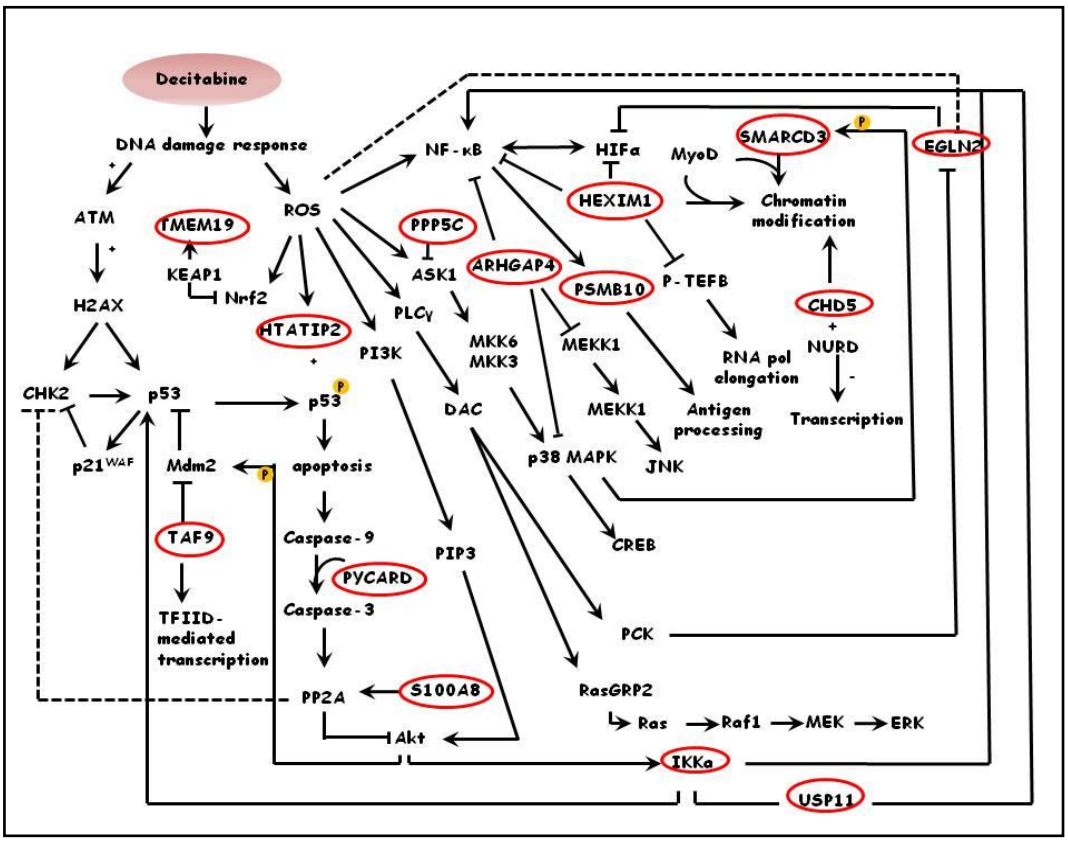


Figure 52, Schematic diagram demonstrating the involvement of 14 shRNA-targeted proteins in the pathways potentially affected by decitabine. The proteins surrounded by a red circle are among the 17 proteins selected for shRNA-mediated knock-downs.

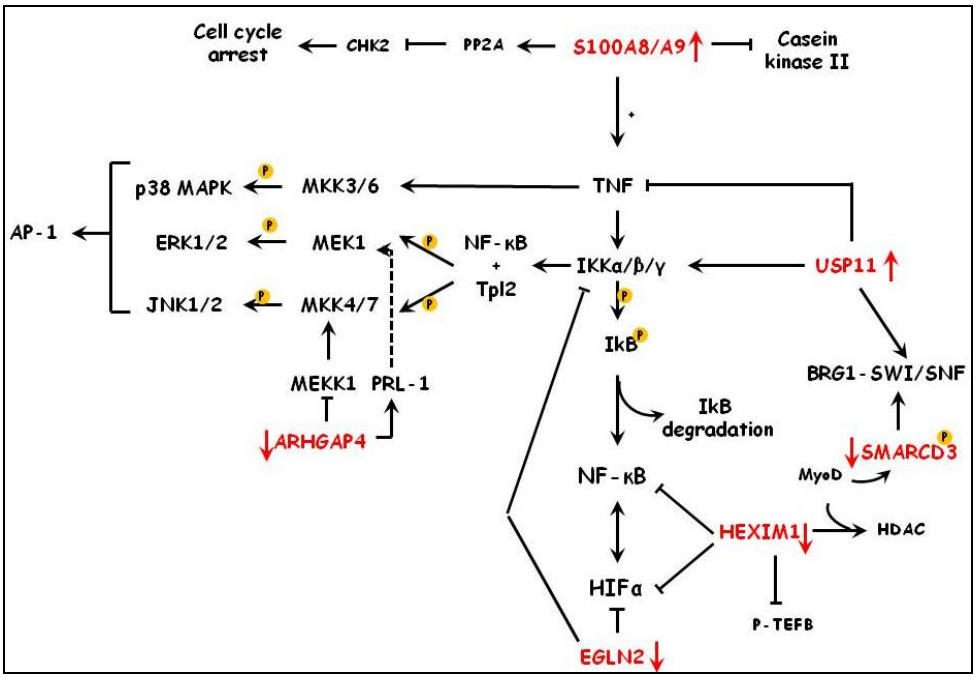


Figure 53, Schematic diagram demonstrating the function of ARHGAP4, EGLN2, HEXIM, USP11, SMARCD3 and S100A8 proteins. The red arrows demonstrate whether the proteins were up- or down-regulated based on the proteomic analysis of primary human erythroid cultures from healthy donors treated with decitabine (ratio 1).

HEXIM1 gene encodes a hexamethylene bisacetamide-inducible protein 1, an inhibitor of positive transcription elongation factor b (P-TEFb) which controls transcription elongation of RNA polymerase II (Lew *et al.*, 2013) (Figure 52 and 53). HEXIM1 directly regulates HIF-1a protein by up-regulating hydroxylation, interacts with pVHL and leads to ubiquitination of HIF-1a. It can also regulate HIF-1a acetylation by attenuating its interaction with HDAC1. Yeh *et al.* (2013) showed that HEXIM1 prevents p53 ubiquitination by competing with HDM2 binding to p53. Besides P-TEFb, HEXIM1 binds to histone deacetylases along with MyoD (Galatioto *et al.*, 2010) and directly interacts with the p65 subunit of NF-κB and subsequently inhibits the transcriptional activity of NF-κB (Ouchida *et al.*, 2003) (Figure 52 and 53). Moreover, Turano *et al.* (2006) demonstrated that induced expression of HEXIM1 leads to terminal differentiation of MEL cells and promotes cell cycle arrest. In the current study, proteomic analysis showed a decrease in HEXIM1 protein expression in healthy cultures treated with decitabine, while *HEXIM1* mRNA levels increased in the presence of decitabine with a parallel increase in HBG expression (Table 25).

S100A8 is a low molecular weight acidic protein with two EF domains and is associated with chronic inflammatory diseases (Passey *et al.*, 1999). Most S100 proteins exist in the form of homodimers or heterodimers within cells and interact with several effector proteins mostly in a Ca^{2+} dependent manner leading to regulation of enzyme activities, the dynamics of cytoskeleton constituents, cell growth and differentiation and Ca^{2+} homeostasis (Donato, 2003). In particular, S100A8/A9 inhibits casein kinase II which is involved in phosphorylation of substrates necessary for cellular transcription and translation such as nuclear oncogenes, RNA polymerase II and topoisomerase (Murao *et al.*, 1989) (Figure 53). In addition, S100A8/A9 complexes stimulate the production of proinflammatory cytokines such as TNFa, a function that is suppressed significantly by p38 MAPK and NF-κB inhibitors (Sunahori *et al.*, 2006) (Figure 53). Moreover, S100A8/A9 down-regulates G2/M cell cycle progression and cell survival by increasing phosphorylation of Cdc2 by PP2A (Khammanivong *et al.*, 2013) (Figure 52). *S100A8* and *S100A9* were among the genes found to be up-regulated significantly in erythroid precursors in patients with sickle cell disease following treatment with hydroxyurea (Costa *et al.*, 2007). In the current study, proteomic analysis showed lower expression of the S100A8 in treated thalassaemic cultures when compared to treated healthy cultures. Down-regulation of *S100A8* has a minimal affect the γ-globin gene levels. However, treatment with decitabine resulted in up-regulation of both *S100A8* and *HBG* expression (Table 25), suggesting the activation of targets bypassing S100A8 might lead to γ-globin induction.

SMARCD3 (Baf60c) is a subunit of a chromatin remodelling complex known as SWI/SNF, that utilises energy from ATP to dissociate DNA-histone contacts therefore providing access to chromatin of silent loci. During myogenic differentiation, BAF60c is phosphorylated by p38α kinase, promoting the incorporation of MyoD-BAF60c complex into a Brg1-based SWI/SNF complex, remodelling of chromatin and subsequent activation of transcription of MyoD-target genes (Forcales, 2012) (Figure 53). In addition, BAF60c physically interact with PPARγ and BRG1 *in vivo* along with other nuclear receptors and transcription factors leading to recruitment of SWI/SNF complex and induction of remodelling of their target genes (Debril *et al.*, 2004). Here we show that down-regulation of *SMARCD3* slightly reduced *HBG* gene expression (Table 24). Upon stimulation with decitabine, SMARCD3 expression dropped significantly while maintaining or increasing slightly γ-globin expression (Table 25), suggesting a subtle inhibitory role of *SMARCD3* gene on γ-globin gene expression.

Our results show poor correlation between gene transcription levels with protein levels. Despite the development of methods for global gene expression analysis, it has been shown experimentally that the mRNA level of a particular gene does not reflect the amount of the corresponding protein expressed in the same cells (Anderson and Seilhamer, 1997, Gygi *et al.*, 1999b). This was previously attributed to the inability of mRNA transcripts to predict post-transcriptional modifications that affect the protein abundance in the cells (Vogel *et al.*, 2010, Vogel and Marcotte, 2012). Proteins are therefore considered as the direct effectors of cellular behaviour rather than their DNA and mRNA templates. For some proteins, constant translation is not required due to their high stability. Thus it is possible that the long half life of proteins in addition to the low proliferating capacity of the primary human erythroid cultures at the end of the experiment could explain the variability in expression between mRNA and protein levels. Although characterisation of protein steady state level provides the most immediate assessment of cellular function capacity, differences between different states of biological systems, i.e. presence and absence of decitabine, cannot be explained just by simply looking at single genes and corresponding proteins. The function and behaviour of biological systems are mainly governed by the interaction among proteins. Many of these interaction that control cellular behaviour are dependent on post-translational modifications induced by cross-talk between canonical signalling pathways.

Based on the above, further functional studies are required for confirmation of the effect of each protein on γ-globin expression in the presence of decitabine. In addition, the function of the above proteins has to be investigated further using second generation proteomics and interactomics in the presence of decitabine. This could provide some insights on the role of decitabine in the post-translational modifications and on protein-protein interactions of the above proteins.

4. CONCLUSIONS AND DISCUSSION

4.1. Conclusions

Despite the fact that >70 compounds have been described as HbF inducers, their clinical use is limited due to low HbF inducing activity, high cytotoxicity and inability to elicit a response in all patients. Thus, identification of new agents with higher efficacy and reduced toxicity is imperative. In the current project two approaches have been employed with the aim of identifying potential HbF inducers. Initially, agents that are structurally similar to compounds with known HbF inducing activity were screened for identification of novel agents with potent HbF inducing activity. The second approach involved the investigation of the molecular pathways of a known HbF inducer with the aim of identifying potential targets for therapeutic manipulation and target based drug design. The conclusions of the current project are as follows:

1. **None of the eleven xanthines tested are effective inducers of foetal haemoglobin**

Based on the observation that intracellular levels of cAMP and phosphorylated CREB levels correlated with foetal haemoglobin in individual patients (Bailey *et al.*, 2007) and that caffeine-mediated increase of Ca^{2+} activated CREB through the binding of Ca^{2+}/cAMP response element in the promoter of CREB-dependent genes (Connolly and Kingsbury, 2010), eleven xanthines including caffeine were investigated as potential HbF inducers in murine and human erytholeukaemic cell lines. Most of the xanthines tested lacked HbF inducing activity. Only two xanthines (Zaprinast and PSB11) increased γ-globin promoter activity but this was accompanied by an increase in cytotoxicity of the compounds. As a result, none of the xanthines tested were appropriate HbF inducers.

2. **Resveratrol and its nine derivatives are not effective in reactivating foetal haemoglobin in primary human erythroid cultures despite the Hb inducing activity shown in K562 cell line.**

Apart from its antioxidant activities, resveratrol was found to inhibit ribonucleotide reductase more efficiently than HU, a bench mark HbF inducer (Ware and Aygun, 2009). Resveratrol has also been shown to stimulate the expression of γ-globin gene and

foetal haemoglobin (HbF) (Fibach *et al.*, 2012). We therefore investigated the role of resveratrol and nine of its derivatives in K562 cells and primary human erythroid cultures as potential HbF inducers. Despite promising results for resveratrol and three derivatives (P1, P4 and P11) in K562 cell line, none of the agents showed an ability to increase HbF levels in primary human erythroid cultures. In fact, resveratrol was shown to accelerate erythroid maturation rather than increasing the expression of the γ-globin gene. In order to enhance the HbF-inducing activity of these agents, we investigated the use of resveratrol in combination with another agent as a potential therapeutic approach. The combined use of resveratrol and decitabine increased the percentage of haemoglobin containing cells when compared to each agent alone but did not surpass the additive effect of the agents. The small increase obtained by the combination in addition to the non-significant change in cytotoxicity above the additive effect of the agents, suggests that the combined administration of two agents is a potential pharmacological approach to enhance their HbF-inducing activity.

3. Decitabine was the most efficient HbF inducing agent among the four pre-selected agents in both K562 cell line and primary human erythroid cultures

Insufficient HbF induction is achieved with current pharmacological agents. In addition, none of the mechanisms of action of these agents is fully understood. We thus selected four HbF inducers (mithramycin, decitabine, angelicin and lenalidomide) and screened them in K562 cells and in primary human erythroid cultures to identify the most potent HbF inducer for delineation of its molecular mechanism of action. Screening of the four known HbF inducers in K562 cells indicated that decitabine was the agent with the highest HbF-inducing activity, while exhibiting moderate cytotoxicity. This was confirmed in primary human erythroid cultures from healthy and thalassaemic donors. Based on our results and the fact that the agent is already in use in clinical trials for the treatment of haematologic cancers, decitabine was selected for further investigation.

4. HbF induction in the presence of decitabine does not depend on the hypomethylating activity of the agent on the γ-globin promoter

Since decitabine is a DNA methyltransferase inhibitor; we began characterising the effect of the agent on γ-globin reactivation by investigating the decitabine-mediated changes in DNA and histone methylation patterns of the β-globin locus. Methylation at Lysine 4 of H3 (H3K4me2) represents open chromatin structure and transcriptional activation. H3K4me2 patterns on the β-globin locus in primary human erythroid cultures grown in the absence of decitabine demonstrated low transcriptional activity at the globin gene promoters and an increased activity at the LCR. Low H3K4me2 at the γ-globin gene promoter with higher H3K4me2 at the β-globin promoter and LCR coincide with the developmental regulation of globin gene expression by the LCR (Figure 54). Treatment of primary human erythroid cultures with decitabine increased H3K4me2 levels at β-HS1 and β-HS4 but decreased β-HS3 and β-HS5 patterns, maintained similar levels at the γ-globin gene promoter and decreased H3K4me2 at the β-globin promoter. These findings suggest that modulation of globin gene expression occurs mainly through the HS sites rather than by direct action on the globin gene promoters (Figure 54). A similar pattern to histone methylation was observed for DNA methylation, with low MeCP2 binding at all globin gene promoters and high binding at the LCR. These DNA methylation patterns were in complete contrast to the function of MeCP2 as a transcriptional repressor, suggesting that MeCP2 acts as an activator rather than a repressor of transcription. DNA methylation patterns should be verified using an alternative method such as bisulphite sequencing.

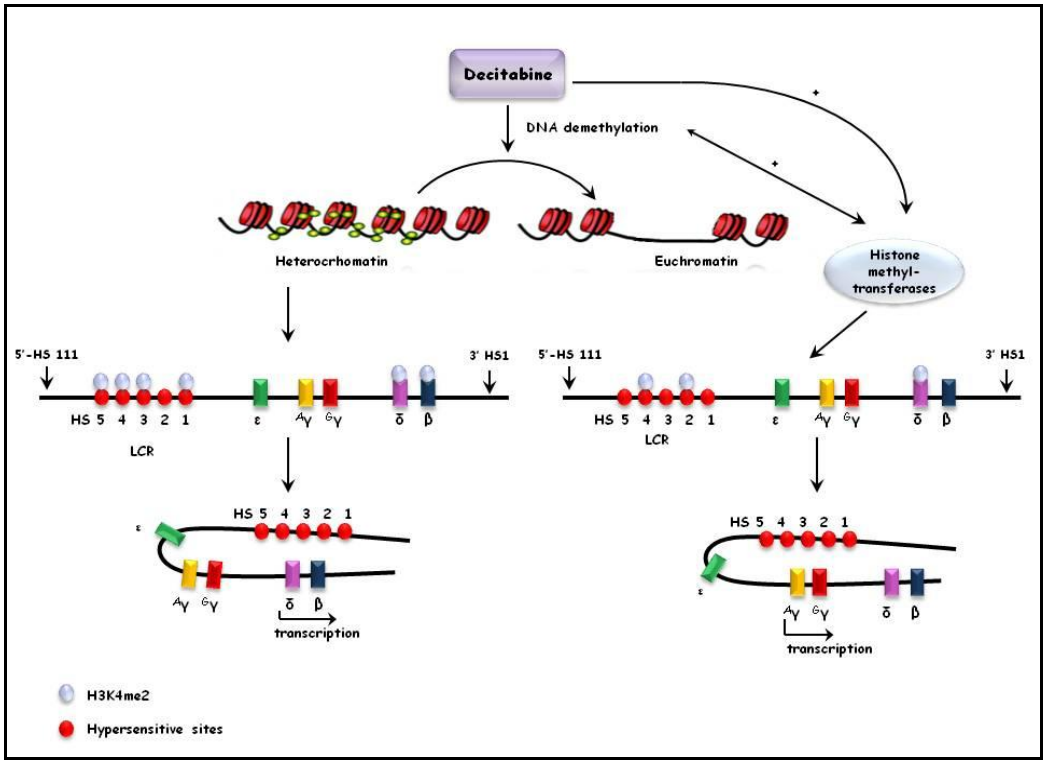


Figure 54, Schematic diagram demonstrating the effect of decitabine on DNA and histone methylation in primary human erythroid cultures from healthy donors. In the absence of decitabine, moderate levels of H3K4me2 are found at the adult β-globin genes (δ and β) and LCR, suggesting an LCR-mediated regulation of globin gene expression. Administration of decitabine leads to DNA demethylation with subsequent recruitment of histone methyltransferases that increase H3K4me2 at HS2 and HS4 of the LCR. The decreased H3K4me2 at the β-globin gene was confirmed by the decrease in β-globin expression. The increase in γ-globin gene expression is speculated to be through interaction with the LCR.

5. Despite the lack of statistical significance, decitabine has a global effect on gene expression of erythroid-related genes in primary human erythroid cultures. Healthy and thalassaemic cultures respond differently to the agent.

Decitabine increased the expression of almost all erythroid-related genes under investigation in healthy cultures after 3 days of treatment, suggesting a global response to the agent. In contrast, decitabine decreased gene expression levels of *BCL11A* and *GATA1* in thalassaemic cultures after 3 days of treatment. However, after 6 days of treatment, the gene expression levels of these genes returned to baseline levels. By classifying the individual cultures into high and low responders based on their HbF response to decitabine, we could demonstrate changes in mRNA levels of different genes in healthy and thalassaemic cultures. *KLF1* was among the genes with the highest expression in high responders both in healthy and thalassaemic cultures after 6 days of

treatment, whereas *MBD2* was among the genes with the highest expression in low responders of both groups after 6 days of treatment with decitabine. The increase in *MBD2* gene expression suggests stimulation of methylation which is not consistent with the function of decitabine as a hypomethylating agent but coincides with the effect observed by ChIP analysis. Differences in gene expression between high and low responders were more prominent in thalassaemic than in healthy cultures (Figure 55). Increasing length of treatment of thalassaemic cultures with decitabine increased *KLF1*, *MBD2* and *CREB* gene expression in high responders. In contrast, increasing length of treatment in low responders of thalassaemic cultures increased *BCL11A* and *GATA1* gene expression, but reduced *CREB* and *HBG* expression (Figure 55). Due to the lack of statistical significance of the above changes, further investigation into the effect of decitabine on gene expression of the above genes is necessary.

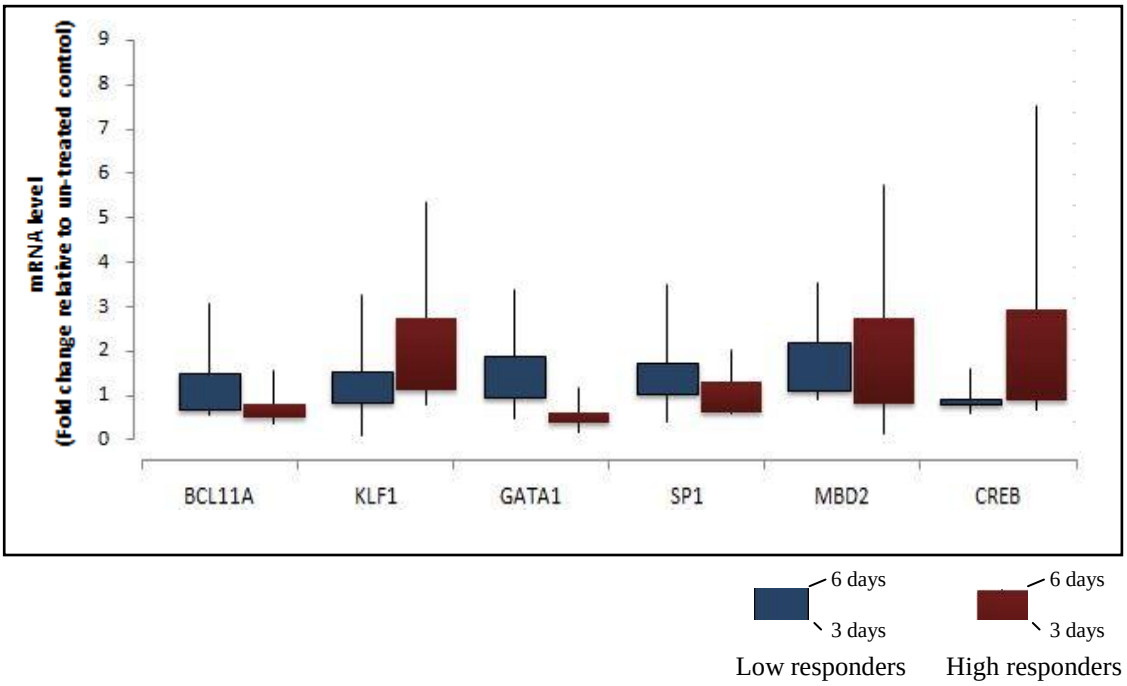


Figure 55, Low and high responders of thalassaemic cultures respond differently to decitabine treatment as shown by the changes in mRNA levels of different genes. The graph demonstrates some of the major changes between low responders and high responders in thalassaemic cultures. Low responders (blue bars) raised *BCL11A* and *GATA1* gene expression after 6 days of treatment above the levels observed after 3 days with the agent, while maintained similar levels of *CREB* gene expression relative to un-treated control after both 3 and 6 days with decitabine. In contrast, high responders (red bars) raised *KLF1*, *MBD2* and *CREB* expression substantially, after 6 days of treatment. Both groups increased the levels of *SP1* expression following 6 days with decitabine, despite differences in the level of expression. However, none of the changes are statistically significant according to the paired t-test (p-value>0.05). Each bar represents the fold change in mRNA levels after 3 (bottom border) and 6 days of treatment (top border).

6. Decitabine induces signal transduction pathways that might lead to foetal globin gene activation

Proteomic analysis of primary human erythroid cultures from transfusion-dependent thalassaemic patients in the presence and absence of decitabine indicated that decitabine promotes survival of immature erythroid progenitors in thalassaemic cultures by potentially modulating erythropoiesis (Figure 40, page 172). In healthy cultures, decitabine-mediated oxidative stress might lead to activation of signal transduction pathways that subsequently cause activation of γ-globin expression (Figure 38, page 169). Functional annotation of differentially expressed proteins in healthy and thalassaemic cultures in the presence of decitabine demonstrated that decitabine modulates protein binding, transcription and chromatin structure in both groups. In addition, some of the differentially expressed proteins in both healthy and thalassaemic cultures are involved in the NF-κB pathway and HIF signalling pathways. Activation of γ-globin expression by decitabine might be a down-stream effect of the activation of these signalling pathways. Further investigation of the effect of these pathways on γ-globin activation and their modulation by decitabine is necessary to confirm such a hypothesis.

7. Decitabine modulates the expression of *ARHGAP4, EGLN2, SMARCD3* and *USP11* genes which are potentially associated with γ-globin expression

Lentiviral shRNA-mediated knock-downs of 17 proteins in primary human erythroid cultures demonstrated the possible association of *ARHGAP4*, *EGLN2*, *SMARCD3* and *USP11* genes with γ-globin expression in the presence of decitabine. Down-regulation of *ARHGAP4*, a RhoGTPase activating protein, resulted in reduced *HBG* expression both at the mRNA and protein levels. Decitabine treatment increased HBG protein expression while maintained a reduced *ARHGAP4* gene expression. In contrast to the increased mRNA levels in un-transduced cultures, proteomic analysis showed a decreased expression of ARHGAP4 protein in the presence of decitabine, suggesting that gene expression correlates inversely with γ-globin expression. Down-regulation of *ELGN2*, a prolyl-3-hydroxylase responsible for post-translational modification of prolines on HIF proteins, resulted in an increased *HBG* expression in the absence of decitabine. In the presence of decitabine, HBG expression was further increased while

maintaining a reduced *EGLN2* expression. These observations suggest that EGLN2 has an inhibitory role on γ-globin expression. Down-regulation of the EGLN2 gene was confirmed by proteomic analysis in healthy and thalassaemic cultures in the presence of decitabine. *SMARCD3* was the only gene whose expression significantly decreased in the presence of decitabine. The decrease in *SMARCD3* expression was accompanied by an increase in HBG expression, again suggesting that the gene inhibits γ-globin expression. Finally, USP11, a de-ubiquitinating enzyme, was the only gene whose expression was profoundly increased by decitabine both in un-transduced healthy cultures at the mRNA and protein levels and in transduced cultures. The increase in USP11 expression was accompanied by a prominent but not statistically significant increase in γ-globin gene expression suggesting a direct role of the gene on γ-globin expression. Although the findings are sometimes contradictory between mRNA and protein levels, they suggest a potential role of these genes in the regulation of γ-globin expression and thus merit further investigation.

4.2. Discussion

Pharmacological reactivation of foetal haemoglobin is a promising therapeutic approach for β-thalassaemia, as foetal haemoglobin can substitute for the absent adult β-haemoglobin. Traditionally, most HbF inducers have been identified based on their ability to alter local promoter chromatin (DNA methyltransferase inhibitors and Histone deacetylase inhibitors) or the kinetics of erythroid differentiation (cytotoxic agents). However, observations such as the ability of 5-azacytidine to increase HbF synthesis in the absence of global DNA methylation, the failure of γ-globin promoter hypomethylation by shRNA-mediated DMNT1 knock-down to induce expression of the gene (Mabaera *et al.*, 2008a), the ability of drugs to induce HbF *in vitro* without changes in the differentiation kinetics (Mabaera *et al.*, 2008a), the ability of short chain fatty acids that do not affect histone acetylation to induce HbF synthesis (Boosalis *et al.*, 2001) and the ability of p38 MAPK inhibitors to block γ-globin and HbF induction by agents (Witt *et al.*, 2000), has raised questions about the molecular mechanism of action of these agents. Over the past few years, groups have redirected their research towards cell signalling as a way to regulate HbF induction. The pathways that have been demonstrated to be involved in γ-globin reactivation, so far, include cyclic guanosine monophosphate and cyclic adenosine monophosphate, nitric oxide, p38 MAPK, ROS

and cytokine signalling. Although each of these signalling pathways has strong evidence about their interaction with HbF induction, they do not explain how such a diverse group of compounds can have the same effect.

In the current study, due to the inability to identify novel potent HbF inducers through screening of agents that are structurally or mechanically associated to known HbF inducers, an alternative approach was employed which involved the delineation of the molecular mechanism of action of decitabine, a known HbF inducer, with the aim of identifying potential targets for design of new agents. Decitabine, a DNA demethylating agent and a safer derivative of 5-azacytidine, was previously shown to increase HbF synthesis efficiently in both baboon and patients with sickle cell disease (Koshy *et al.*, 2000, Saunthararajah *et al.*, 2003, Saunthararajah *et al.*, 2008). In accordance with previous studies, we have demonstrated that decitabine can increase HbF percentage in primary human erythroid cultures from healthy and transfusion-dependent thalassaemic donors. The effect on γ-globin expression was also visible at the mRNA level. This coincides with the findings by Chin *et al.* (2009) which suggested that increased γ-globin expression in baboons involved increased transcriptional activation of the γ-globin gene. More recently, multiple studies have shown that decitabine increases γ-globin expression by post-transcriptional and/or translational mechanisms associated with activation of stress-signal transduction pathways rather than through direct transcriptional activation (Chin *et al.*, 2009, Mabaera *et al.*, 2008a, Mabaera *et al.*, 2008b).

A concurrent increase in the α-globin gene expression was noted in healthy but not in thalassaemic cultures after treatment suggesting a different response to the agent in the presence of the disease state. Despite the general increase in γ-globin expression as demonstrated by all the techniques, a large variation in the response to the agent was observed between individuals. This variation in response was previously demonstrated in relation to other HbF inducers such as HU (Pourfarzad *et al.*, 2013) and was mainly attributed to the presence of HbF-associated SNPs in *BCL11A* gene or to the XmnI restriction site polymorphism of the γ-globin gene promoter (Ronchi and Ottolenghi, 2013, Stamatoyannopoulos, 2005).

Although DNA methyltransferase inhibitors have been known to reactivate HbF for more than 30 years, the mechanism of induction of γ-globin expression by these agents

still remains controversial. Initially, there was evidence that globin gene activation by 5-azacytidine correlated with hypomethylation of a specific CpG site located 53-bp upstream of the γ-globin transcription initiation site (Ley *et al.*, 1983). These findings led to the conclusion that DNA methyltransferase inhibitors increase HbF via reduction in the level of DNA methylation of the γ-globin gene promoter.

Although DNA methylation was not successfully determined in the current study, we suggest that γ-globin induction by decitabine does not depend on hypomethylation of the γ-globin gene promoter but rather due to the LCR-mediated activation of γ-globin gene promoter (Figure 54). This was based on the observations that treatment of primary human erythroid cultures with decitabine led to alterations in the H3K4me2 patterns at the LCR with no significant changes in the γ- and β-globin gene promoters. This pattern, together with the small decrease in the β-globin gene expression observed in primary human erythroid cultures from healthy donors, is consistent with the idea that decitabine increases γ-globin expression through modulation of its interaction with the LCR. Evidence that reduction of DNA methylation of the γ-globin promoter by RNAi targeting DNMT1 was not sufficient to induce γ-globin expression (Mabaera *et al*, 2008b), supports our hypothesis and lead to questions about the actual mode of action of decitabine.

The mechanism of action of decitabine to reactivate foetal haemoglobin in primary human erythroid cells was investigated for the first time here. Post-transcriptional mechanisms that regulate the rate of synthesis and half-life of proteins suggests that the mRNA levels of a particular gene might not always reflect the amount of protein expressed and that measurement of protein levels is crucial. Here, we employed a quantitative proteomic approach for delineation of the molecular function of decitabine in primary human erythroid progenitor cells from healthy and transfusion-dependent thalassaemic donors. Our results demonstrated different roles of decitabine in eliciting HbF induction between healthy and thalassaemic primary human erythroid cultures (Figure 38 and 40). Decitabine promoted survival of immature erythroid progenitors in thalassaemic cultures as well as regulate transcriptional activation of downstream genes in oxidative stress pathways, an effect also shown in healthy cultures. Additionally, cellular stresses including oxidative and ER stress, in healthy cultures might lead to activation of signal transduction pathways that will lead to chromatin remodelling and transcription. Recently, decitabine was shown to induce ROS generation in leukaemia

cells. This response was found to be hypomethylation-independent but rather a consequence of the incorporation of decitabine into DNA and the subsequent activation of signalling pathways due to DNA damage (Fandy *et al.*, 2014) (Figure 56). It might be this DNA damage by decitabine that leads to cellular stresses observed in the healthy primary human erythroid cultures. In addition, Mabaera *et al.* (2008a) proposed that at doses that strongly induce foetal gene expression, 5-azacytidine promotes low-level activation of DNA damage, cell stress or other signalling pathways that lead to the binding of positive-acting transcription complexes to the globin promoters and block binding of DNMT1 and other repressive factors.

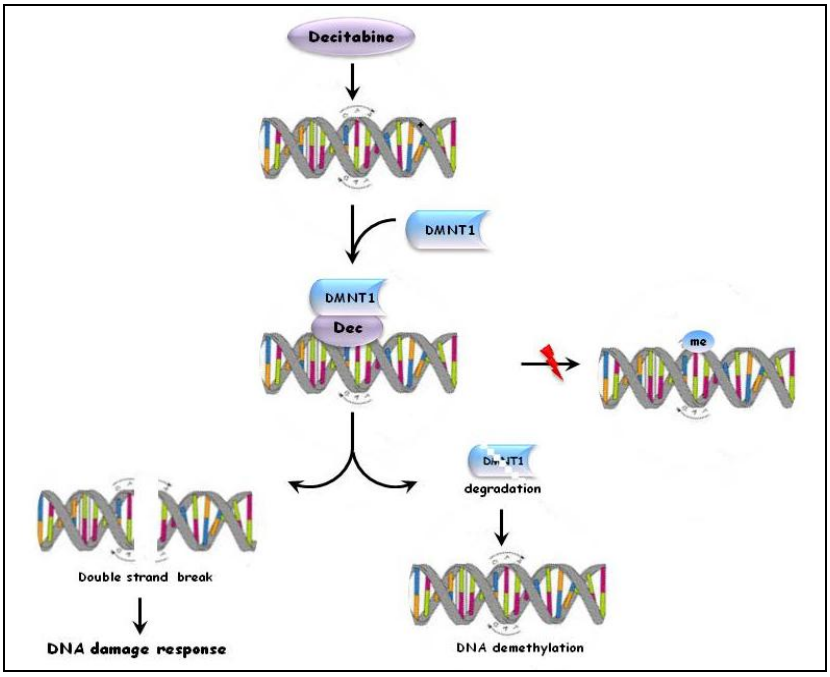


Figure 56, The effect of decitabine incorporation on DNA structure and DNA demethylation. Upon uptake into the cell, decitabine is metabolised into its active form which can bind to DNA as a substitute for cytosine. The metabolised drug is recognised by the DNMT1 that initiates methylation. However, methylation is blocked in the presence of decitabine due to the replacement of carbon-5 of the cytosine ring with nitrogen. This blocks the methyltransferase activity of DNMT1 and triggers DNA damage signalling, leading to degradation of the trapped DNMT1 and formation of double strand breaks.

Comparison of the differentially expressed proteins in healthy and thalassaemic cultures, suggests that decitabine modulates the expression of proteins involved in protein binding, chromatin organization and regulation of transcription. In addition, differentially expressed proteins in both healthy and thalassaemic primary human erythroid cultures treated with decitabine are involved in the regulation of the NF-KB pathway.

Several lines of evidence have suggested that NF-κB plays a crucial role in erythropoiesis. NF-κB subunits p65, p50 and p52 are all expressed during early normal erythroid proliferation and their levels decline during differentiation (Zhang *et al.*, 1998). The three subunits form complexes with κB elements in the promoters of c-myb and c-myc, two oncogenes required in erythroid development suggesting modulation of erythropoiesis by NF-κB factors through regulation of those two genes (Figure 57). Liu *et al.* (2003) demonstrated that activation of the NF-κB pathway represses the expression of the α-like globin genes through repression of the erythroid-specific subunit p45 of NF-E2, a DNA-binding transcription activator that binds to the HS-40 enhancer and the LCR of the β-like globin gene (Figure 57). Recently, resveratrol was shown to inhibit TNFα-mediated NF-κB activation and promote erythropoiesis in primary human erythroid progenitor cells (Jeong *et al.*, 2011). In addition, the immunomodulator thalidomide induces HbF production possibly through suppression of NF-κB induction by inflammatory cytokines such as tumour necrosis factor, a vascular endothelial growth factor associated with increased release of ROS. Therefore, it remains to be determined whether NF-κB modulates γ-globin gene expression through regulation of erythropoiesis by targeting c-myb, c-myc and NF-E2, or NF-κB modulation is a secondary response to cellular stress or upstream signal transduction pathways by decitabine (Figure 58).

Among the differentially expressed proteins, ARHGAP4, EGLN2 and USP11 are involved with the NF-κB pathway, and SMARCD3, in chromatin modulation. Lentiviral-mediated shRNA knock-down of the above genes, demonstrated possible associations of these genes with γ-globin expression in the presence and absence of decitabine.

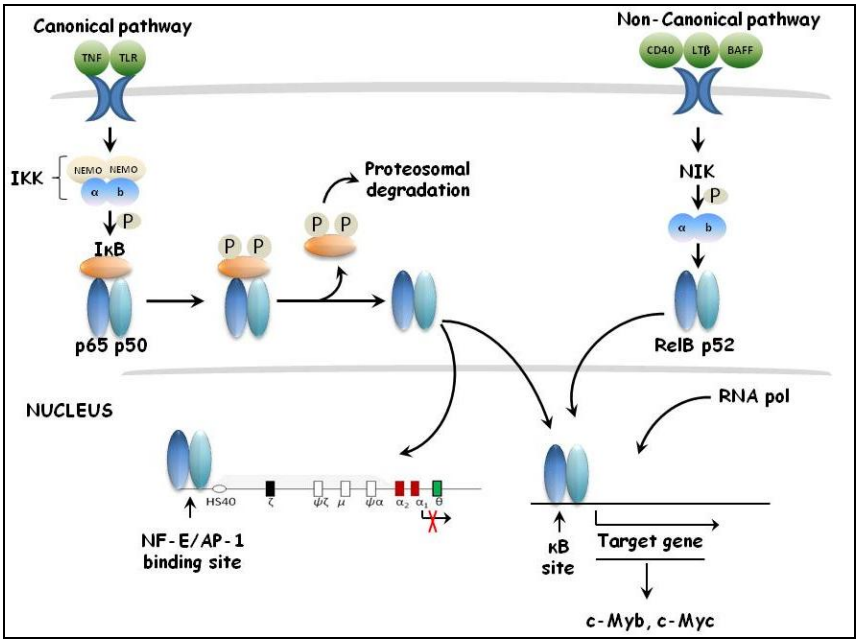


Figure 57, Schematic diagram of the NF-κB signalling pathways. There are two major signalling pathways that lead to activation of NF-κB transcription factor; the canonical (classical) pathway and the non-canonical pathway, each activated in response to different stimuli. Among the down-stream targets of NF-κB activation is the c-Myb and c-Myc transcription factors which are involved in erythropoiesis. In addition, NF-κB inhibits transcription of the α-globin genes through repression of the erythroid-specific subunit p45 of NF-E2.

Proteomic analysis of primary human erythroid cultures treated with decitabine showed a down-regulation of the ARHGAP4 protein in healthy donors. Reduced expression of *ARHGAP4* gene resulted in induction of HBG expression in the presence of the agent suggesting an inverse effect of the gene on γ-globin expression. *ARHGAP4* gene, which encodes for RhoGTPase activating protein 4 (p115 RhoGAP), prevents the association of MEKK1 with components of the c-Jun-N terminal kinase/stress activated protein kinases (JNK) MAP kinase cascade through its SH3 domain (Christerson *et al.*, 2002), promotes ERK1/2 and RhoA activation through its interaction with PRL-1 (Bai *et al.*, 2011) and inhibits MEKK1-mediated activation of transcriptional regulator AP-1 and NF-κB (Christerson *et al.*, 2002). Mardini *et al.* (2010) demonstrated that ERK1/2 MAPK signalling pathway represses globin chain synthesis in MEL cells whereas p38 MAPK pathway signalling acts as positive regulator of globin production. Thus, *ARHGAP4* might activate erythroid differentiation and globin synthesis due to down regulation of ERK signalling pathway.

Proteomic analysis showed that treatment of primary human erythroid cultures from healthy and thalassaemic donors with decitabine down-regulated EGLN2 protein. Western blot analysis showed that primary human erythroid cultures with knocked-down EGLN2 expression had increased HBG expression when treated with decitabine. This suggests an inhibitory effect of EGLN2 on γ-globin expression in the presence of the agent. These findings are in agreement with observations that a reduction in the expression of EGNL2 by PHD inhibitor FG-2216 increased foetal haemoglobin expression in primary human erythroid cells *in vitro* (Hsieh *et al.*, 2007). In addition, reduced EGLN2 expression significantly increased erythropoiesis in rhesus macaques with a moderate increase of HbF-containing red cells (Hsieh *et al.*, 2007). Loss of EGLN2 promotes the accumulation of FOXO3a as a result of enhanced FOXO3a protein stability, the FOXO3a subsequently suppresses cyclin D1 expression which plays a critical role in mammary proliferation (Zheng *et al.*, 2014). Moreover, over-expression of EGLN2 inhibited NF-κB activity through negative regulation of IKKβ (Cummins *et al.*, 2006) while increased the expression of TNF-α, IL-1β and IL-8 significantly, suggesting EGLN2 as a potential inhibitor of NF-κB (Xie *et al.*, 2014).

USP11, a de-ubiquitinating enzyme, is involved in several signalling pathways, including the NF-κB signalling pathway. USP11 gene positively regulates the expression of IKKα expression at the transcriptional level in an ubiquitin-independent manner. In parallel, USP11 was shown to negatively regulate NF-κB activity in response to TNF-α (Yamaguchi *et al.*, 2007a). Following observations that upon oxidative stress, IKKα regulates the transcriptional activity of the p53 tumor suppressor (Yamaguchi *et al*., 2007b), USP11 was shown to be a novel regulator of p53, essential for p53 activation in response to DNA damage (Ke *et al*., 2014). In addition, USP11 has pro-survival functions in cellular response to DNA damage (Schoenfeld *et al*., 2004) with USP11-silenced cells demonstrating defective repair of DNA double-strand breaks by homologous recombination (Wiltshire *et al*., 2010). *USP11* was the only gene whose expression increased profoundly after decitabine treatment in both un-transduced cultures and in the presence of the shRNA. This was confirmed in the proteomic analysis where USP11 was significantly increased in primary human erythroid cultures from healthy donors treated with decitabine suggesting a direct role in γ-globin expression.

The interaction of the above genes with the NF-κB pathway and their possible role in γ-globin expression enhances our hypothesis about the involvement of the above pathway in γ-globin induction by decitabine. However, further investigation is necessary to confirm of the role of the above genes in HbF induction and to determine whether their effect on γ-globin expression is indeed through modulation of the NF-κB pathway.

Due to the fact that several agents including HU, butyrates and thalidomide were found to induce HbF through p38 MAPK signalling pathway, much of the research has been focused towards that pathway. Mabaera *et al.* (2008b) introduced a mechanistic model of HbF induction which integrates the activation of p38 MAPK kinase through multiple upstream signals including DNA damage, oxidative stress (ROS), heat shock, osmotic shock, NO and inhibition of protein synthesis. This was supported by the failure of HbF inducing agents to induce γ-globin expression in the presence of p38 MAPK inhibitor and the ability of constitutively active forms of MKK3 and MKK6, upstream activators of p38 MAPK, to independently increase γ-globin gene expression (Pace *et al.*, 2003). The findings of this study coincide with the stress signalling model proposed by Mabaera *et al.* (2008b). However, the involvement of the p38 MAPK pathway in the mechanism of action of decitabine was not clearly demonstrated by the current findings but instead an involvement of NF-κB pathway was prominent. Whether regulation of the latter pathway is due to down-stream regulation of the p38 MAPK pathway needs to be determined (Figure 58). In addition, further investigation is required to determine whether the activation of the above model is by DNA demethylation of genes involved in stress signalling or whether decitabine does in fact induce apoptosis and DNA damage due to the formation of covalent DNMT-DNA adducts as suggested previously (Juttermann *et al.*, 1994, Palii *et al.*, 2008) (Figure 58). The current project has highlighted some important decitabine-mediated changes in the proteomics of primary human erythroid cultures that will provide the basis of further research into its mechanisms of regulation of HbF production.

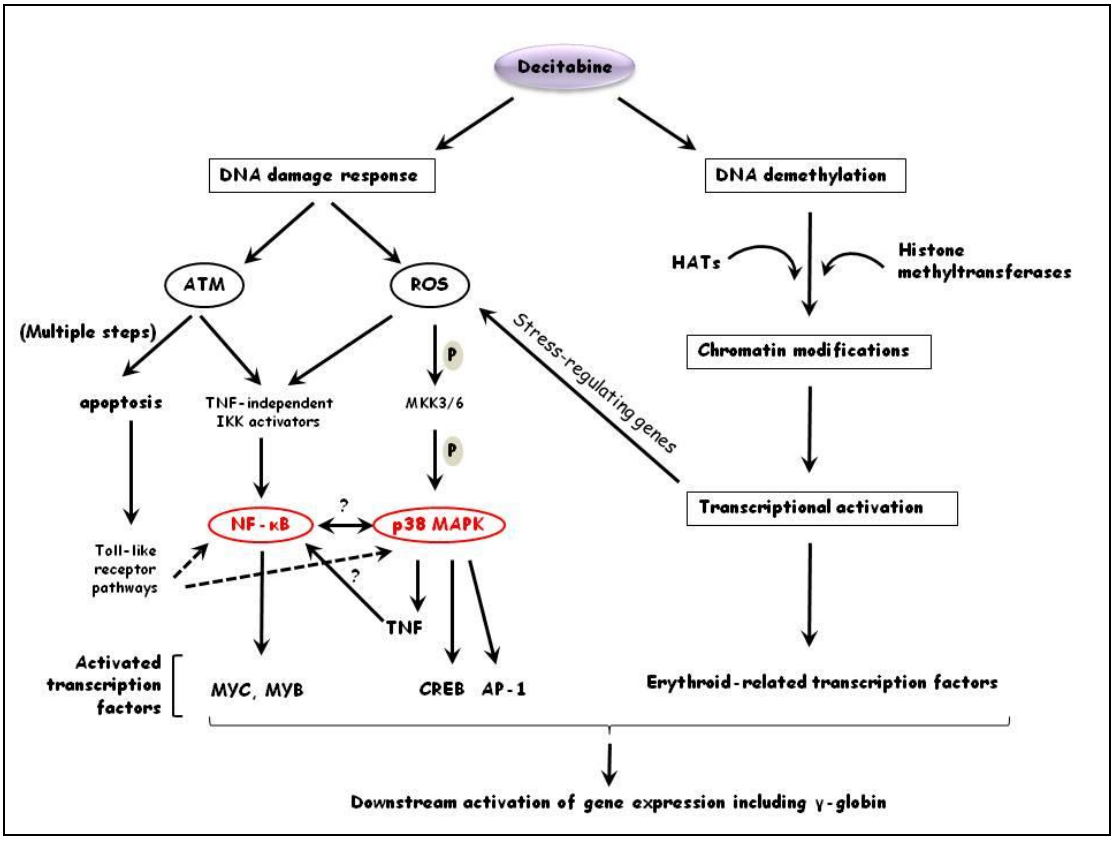


Figure 58, Schematic diagram demonstrating the proposed pathways leading to γ-globin induction in the presence of decitabine.

5. FUTURE WORK

5.1. Analysis of the proteomic data using an alternative statistical model

Existing literature has emphasised that variation can occur throughout the process in the analytical technique, including fluctuations in the autosampler, the use of multidimensional separation, instrument platforms and database algorithms. Moreover, variation in the lists of identified peptides and proteins between replicates can impact the ability of these analyses to accurately represent biological states (Tabb *et al*., 2009). Thus, proper analysis of the proteomic results is of crucial importance in interpreting the results.

One common approach of identifying differentially expressed proteins is to calculate the ratio of the observed peptide intensities between two samples and to compare the calculated ratios against selected upper and lower bounds for ratio thresholds. Hill *et al.* (2008) proposed an ANOVA model which integrates differential expression and normalization of experimental variability in a single analysis. Later, a novel Bayesian model was introduced where observed peptide intensities takes into account both the protein expression levels and peptide effects while taking into consideration the missing peptides (Luo *et al.*, 2009). However, none of the current methods can employ all aspects of variation and missing data in a single analysis. It is thus important to choose the best model based on the particular objectives of the study and recourses available that will lead to the successful interpretation of study results.

It is therefore important to consider the limitations of the iTRAQ approach used in the current study and re-analyse the data using an alternative analysis models. This can lead to new directions about the molecular mechanisms of action of decitabine that need to be considered.

5.2. Investigation of the role of the NF-κB pathway in HbF reactivation and its association with the p38 MAPK signalling pathway

Proteomic analysis of primary human erythroid cultures from healthy and thalassaemic donors treated with decitabine has demonstrated differential expression of proteins that are involved in the NF-κB signalling pathway. Despite identification of potent HbF inducers that can modulate the NF-κB pathway, major focus has been directed on the p38 MAPK due to the ability of a number of diverse HbF inducers to activate HbF

expression via that pathway. We suggest that further investigation should be done to determine whether NF-κB is responsible for modulating γ-globin gene expression in primary human erythroid cultures and whether its effect is through regulation of its downstream targets or whether it is a secondary response to cellular stress or upstream signal transduction pathways that lead to γ-globin gene activation (Figure 58).

Verification of the role of such a pathway will require demonstration of a complete series of molecular events leading to the activation of γ-globin gene expression and increased HbF production. As a first step, investigation of the effect of selective inhibitors of the NF-κB pathway or agonist of negative regulatory molecules within the pathway, on HbF production should be carried out in primary human erythroid progenitor cells. Further verification of the effect can be pursued through the use of knock-down of specific factors along the pathway by short hairpin RNA or by the expression of dominant mutant signalling regulators.

Several studies have shown that p38 MAPK regulates the transcriptional activation of NF-κB transcription factor rendering the NF-κB as a downstream activation of p38 MAPK (Saha *et al.*, 2007, Sun *et al*., 2013). The lack of demonstration of p38 MAPK activation in the current study (despite previous observations by Mabaera *et al*, 2008) and the involvement of the NF-κB pathway in the action of decitabine, necessitates further investigation to determine whether there is an association between the p38 and NF-κB signalling pathways in primary human erythroid progenitor cells.

5.3. Define the role of *ARHGAP4, EGLN2* and *USP11* in HbF production

Reduced expression of *ARHGAP4* and *EGLN2* genes increased HbF expression in primary human erythroid cultures treated with decitabine suggesting an inhibitory role of those genes on HbF production under the regulation of decitabine. In addition, *USP11* expression was increased in the presence of decitabine along with a large increase in γ-globin expression suggesting a positive regulation of HbF by *USP11*. The role of these genes in HbF production should be investigated further by functional studies. We first need to confirm whether the results are consistent as the above findings are the outcomes of a single experiment. Moreover, investigation of the baseline expression of these genes prior to treatment in primary human erythroid cultures is also

necessary for confirmation of the original experiments. Finally, future work will determine the effect that these genes have on HbF expression in primary human erythroid cultures from peripheral blood of patients treated with decitabine.

5.4. Investigation of the role of all differentially expressed proteins in HbF induction by decitabine

Due to time constrains, not all of the 27 differentially expressed proteins associated with the action of decitabine identified through proteomic analysis could be investigated further. With the possibility of losing important proteins during selection, only 6 out of the 27 proteins were investigated for their role in HbF induction with and without decitabine. It is therefore important to investigate the effect of all of the differentially expressed proteins in HbF induction in the presence and absence of decitabine. Lentiviral-mediated shRNA knock-downs should be employed as a first attempt to define their effect on γ-globin expression and their response to decitabine. Interesting proteins could then be investigated further using functional studies.

5.5. Validation of current data and further analysis of methylation patterns in primary human erythroid cultures in the absence and presence of decitabine

The role of hypomethylation by decitabine and 5-azacytidine in the reactivation of foetal haemoglobin has been controversial among different studies (Chin *et al*., 2009, Saunthararajah *et al.*, 2004, Mabaera *et al*., 2008a). Furthermore, most of the investigations on hypomethylation of the γ-globin promoter were done in baboons. Although the baboon is an important animal model due to the almost identical structure of the β-globin locus and developmental pattern of expression of genes within the locus to man (DeSimone and Mueller, 1978), differences in mechanisms of the transcriptional activation of γ-globin gene expression were observed between decitabine-treated baboons and erythroid progenitor cell cultures from decitabine-treated baboon bone marrow. As a result, confirmatory experiments will need to be performed in human primary human erythroid progenitor cells.

Despite attempts to investigate changes in DNA methylation patterns in primary human progenitor cells before and after treatment with decitabine in the current study, it was

not possible to determine the methylation patterns at the β-globin locus accurately. The investigation should be repeated in order to determine the effect of decitabine on the β-globin locus in such a system following treatment with the agent using more appropriate technique. Bisulphite sequencing remains the gold standard for DNA methylation analysis and provides readout of the methylation status of individual cytosines within a defined region of the genome leading to identification of differentially methylated DNA species within a population, a property that is not achieved by other techniques.

5.6. Transcriptomic analysis of the effect of the agent in decitabine-treated patients

A global protein profiling approach provides a common platform for the simultaneous detection of thousands of drug-related changes in proteins. However, when the drug is believed to act via regulation of transcription as in the case of decitabine, it might be more appropriate to investigate the drug-mediated changes at the gene expression level. This can be done by the use of microarrays for the simultaneous measurement of expression levels of a large number of genes. On a higher level, RNA sequencing would not only be qualitative but also quantitative in determining changes in gene expression of potential transcription factors that are involved in globin gene expression.

In addition, observations from the peripheral blood of patients treated with decitabine may be more representative of the clinical effects than treatment of primary human erythroid cells *in vitro*. The use of high levels of foetal bovine serum and growth factors in the *in vitro* model might affect the action of the drug. Therefore, transcriptomic analysis (RNA) and proteomic analysis should be employed to investigate the effect of the agent in peripheral blood isolated from decitabine treated patients.

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APPENDIX I

Table I.I, List of all the agents identified as HbF inducers so far

	Agent	*Reference*
DNA methyltransferase inhibitors	5-azacytidine	De Simone *et al.* (1982), Ley *et al.* (1982)
	Decitabine	Sauthararajah *et al.* (2003)
	5,6 dihydro-5-azacytidine	Carr *et al.* (1987)
	S110	Lavelle *et al.* (2007)
DNA alkylators	Busulfan	Liu *et al.* (1990)
	Cisplatin	Bianchi *et al.* (2000)
	Streptozotosin	Iyamu *et al.* (2000)
Nucleoside analogue	Cytosine arabinoside	Veith *et al.* (1985)
Inosine monophospate dehydrogenase Inhibitors	Ribavirin	Yu *et al.* (1989)
	Mycophenolic acid	Yu *et al.* (1989)
	Tiazofulin	Yu *et al.* (1989)
Ribonucleotide reductase inhibitor	Didox	Pace *et al.* (1994)
	Hydroxyurea	Letvin *et al.* (1984), Charache *et al.* (1987)
	Resveratrol	Rodrigue *et al.* (2001)
	Trimidox	Iyamu *et al.* (1998)
DNA intercalating agents	Aclarubicin	Delgado-Canedo *et al.* (2005)
	Chromomycin	Bianchi *et al.* (1999)
	Distamycin	Bianchi *et al.* (2001)
	Doxorubicin	Delgado-Canedo *et al.* (2005)
	Mithramycin	Bianchi *et al.* (1999)
	Tallimustin	Bianchi *et al.* (2001)
Psoralens	Angelicin	Lampronti *et al.* (2003)
	5-Metyhoxypsoralen	Viola *et al.* (2008)
	Trimethyl angelicin	Lampronti *et al.* (2003)
Dihydrofolate reductase inhibitor	Methotrexate	Veith *et al.* (1989)
Microtubule inhibitor	Vinblastine	Veith *et al.* (1985)
Protein synthesis inhibitor	Anisomycin	Pace *et al.* (2003)
Short chain fatty acids	Butyrate	Ginder *et al.* (1985), Perrine *et al.* (1985)
	Phenyl butyrate	Fibach *et al.* (1993)
	α-aminobutyric acid	Constantoulakis *et al.* (1988)
	2-methylbutyric acid	Pace *et al.* (2002)
	Tributyrin	Witt *et al.* (2000)
	Acetate	Stamatoyannopoulos *et al.* (1994)
	Phenylacetate	Fibach *et al.* (1993)
	Phenoxyacetic acid	Torkelson *et al.* (1996)
	Butyryl-hydroxamate	Cao *et al.* (2005)
	α-Methylhyrocinnamic acid	Torkelson *et al.* (1996)
	Caproate	Safarya *et al.* (1994)
	Heptanoic acid	Liakopoulou *et al.* (1995)
	Hexanoic acid	Liakopoulou *et al.* (1995)
	Isobutyramide	Perrine *et al.* (1994)
	Nonanoic acid	Liakopoulou *et al.* (1995)
	Octanoic acid	Liakopoulou *et al.* (1995)
	Pentanoic acid	Liakopoulou *et al.* (1995)
	Propionic acid	Liakopoulou *et al.* (1995)
	Propional hydroxamate	Skarpidi *et al.* (2003)
	Dimethyoxyphenyl proprionic acid	Pace *et al.* (2002)
	RB7	Mankidy *et al.* (2006)
	RB4, RB9, RB29	Bohacek *et al.* (2006)

	Valproic acid	Collins *et al.* (1994)
Histone deacetylase inhibitors	Apicidin	Witt *et al.* (2003)
	FK228	Cao and Stamatoyannopoulos (2006)
	Compounds 24 and 29	Mai *et al.* (2007)
	Helminthsporium toxin	McCaffrey *et al.* (1997)
	MS-275	Witt *et al.* (2003)
	SAHA (Vorinostat)	Skarpidi *et al.* (2003)
	SBHA	Skarpidi *et al.* (2003)
	Scriptaid	Johnson *et al.* (2005)
	Trapoxin	McCaffrey *et al.* (1997)
	Trichostatin A	McCaffrey *et al.* (1997)
Immunomodulatory drugs	Thalidomide	Aerbajinai *et al.* (2007)
	Lenalidomide (Revlimid)	Moutouh-de Parseval *et al.* (2008)
	Pomalidomide	Moutouh-de Parseval *et al.* (2008)
Hormonal agents	Nomegestrol	Nascimento *et al.* (1998)
	Progesterone	da Silva Santo Duarte *et al.* (2002)
Cytokines	Erythropoietin	Al-khati *et al.* (1987)
	Stem cell factor	
	TGF-β	Bohmer (2003)
mTOR inhibitors	Rapamicin	Fibach *et al.* (2006)
	Everolimus	Zuccato *et al.* (2007)
5-lipoxygenase inhibitor	Zileuton	Haynes *et al.* (2004)
Phosphatase inhibitor	Vanadate	Amoyal *et al.* (2007)
HIF-prolyl hydroxylase inhibitor	FG-2216	Hsieh *et al.* (2007)
Nitric oxide donor	CysNO	Cokic *et al.* (2003)

APPENDIX II

Table II.I, List of the shRNA obtained from the TRC Mission Library for knocking-down the proteins under investigation.

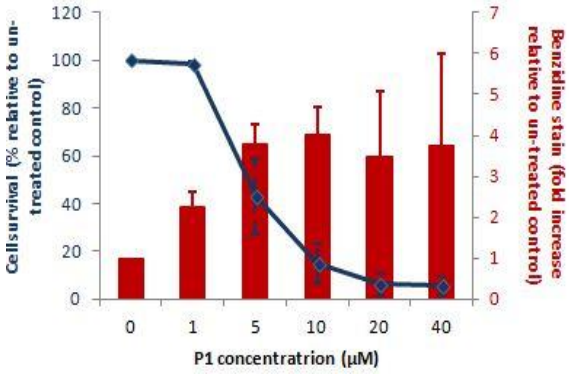
TRC clone ID	*Gene*	*Oligo sequence*	*Target region*	*Clone Name*	*Clone Number*
TRCN0000002800	PPP5C	CCGGCCAGATCACTTTCACCTCCTTCTCGAGAAGGAGGTGAAAGTGATCTGGTTTTT	CDS	NM_006247.x-940s1c1	1
TRCN0000002801	PPP5C	CCGGCCACGAGACAGACAACATGAACTCGAGTTCATGTTGTCTGTCTCGTGGTTTTT	CDS	NM_006247.x-969s1c1	2
TRCN0000002802	PPP5C	CCGGGAGACAGAGAAGATTACAGTACTCGAGTACTGTAATCTTCTCTGTCTCTTTTT	CDS	NM_006247.x-757s1c1	3
TRCN0000002803	PPP5C	CCGGGAAGAGAACAACCTGGACTATCTCGAGATAGTCCAGGTTGTTCTCTTCTTTTT	CDS	NM_006247.x-1306s1c1	4
TRCN0000002804	PPP5C	CCGGGAAGTACATCAAGGGTTATTACTCGAGTAATAACCCTTGATGTACTTCTTTTT	CDS	NM_006247.x-339s1c1	5
TRCN0000007358	USP11	CCGGCCCTCCCTTCTAGTCTTTATTCTCGAGAATAAAGACTAGAAGGGAGGGTTTTT	3UTR	NM_004651.2-3123s1c1	1
TRCN0000007359	USP11	CCGGCCGTGATGATATCTTCGTCTACTCGAGTAGACGAAGATATCATCACGGTTTTT	CDS	NM_004651.2-1695s1c1	2
TRCN0000007360	USP11	CCGGCCGATTCTATTGGCCTAGTATCTCGAGATACTAGGCCAATAGAATCGGTTTTT	CDS	NM_004651.2-653s1c1	3
TRCN0000011089	USP11	CCGGCGGCACAATGATTTGGGCAAACTCGAGTTTGCCCAAATCATTGTGCCGTTTTT	CDS	NM_004651.2-607s1c1	4
TRCN0000011090	USP11	CCGGCCGTGACTACAACAACTCCTACTCGAGTAGGAGTTGTTGTAGTCACGGTTTTT	CDS	NM_004651.2-1800s1c1	5
TRCN0000062023	TACC3	CCGGCCACGGAGCCGCTGTCCCCGCCTCGAGGCGGGGACAGCGGCTCCGTGGTTTTTG	3UTR	NM_006342.1-2629s1c1	1
TRCN0000062024	TACC3	CCGGGCAGTCCTTATACCTCAAGTTCTCGAGAACTTGAGGTATAAGGACTGCTTTTTG	CDS	NM_006342.1-1776s1c1	2
TRCN0000062025	TACC3	CCGGCGCACAGGATTCTAAGTCCTACTCGAGTAGGACTTAGAATCCTGTGCGTTTTTG	CDS	NM_006342.1-305s1c1	3
TRCN0000062026	TACC3	CCGGGCTTGTGGAGTTCGATTTCTTCTCGAGAAGAAATCGAACTCCACAAGCTTTTTG	CDS	NM_006342.1-1905s1c1	4
TRCN0000062027	TACC3	CCGGCCAGGAAGTTCTGAGAACCAACTCGAGTTGGTTCTCAGAACTTCCTGGTTTTTG	CDS	NM_006342.1-589s1c1	5
TRCN0000146887	SMARCD3	CCGGCCATGACAAGGAATACATCAACTCGAGTTGATGTATTCCTTGTCATGGTTTTTTG	CDS	NM_003078.2-1020s1c1	1
TRCN0000147806	SMARCD3	CCGGGAAACTGGATCAAACCATCATCTCGAGATGATGGTTTGATCCAGTTTCTTTTTTG	CDS	NM_003078.2-513s1c1	1
TRCN0000147991	SMARCD3	CCGGGACAAGTATTTCCAGCAGATTCTCGAGAATCTGCTGGAAATACTTGTCTTTTTTG	CDS	NM_003078.2-1045s1c1	2
TRCN0000179523	SMARCD3	CCGGGCGGAAGTTCTCTTCTTTCTTCTCGAGAAGAAAGAAGAGAACTTCCGCTTTTTTG	CDS	NM_003078.2-717s1c1	3
TRCN0000180907	SMARCD3	CCGGGCTCTGGACAGTAAGATCCATCTCGAGATGGATCTTACTGTCCAGAGCTTTTTTG	CDS	NM_003078.2-1282s1c1	4

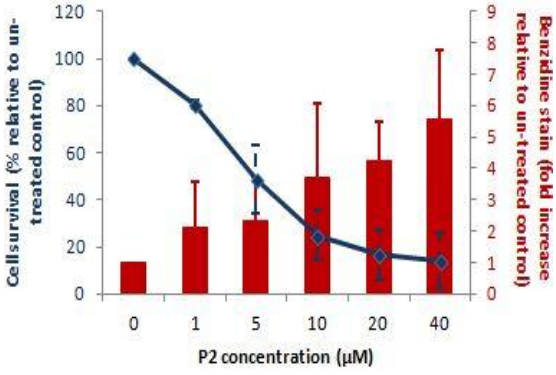
TRCN0000003935	PSMB10	CCGGGATCACAAAGACTGGCGCCAACTCGAGTTGGCGCCAGTCTTTGTGATCTTTTT	CDS	NM_002801.x-749s1c1	1
TRCN0000003936	PSMB10	CCGGCGGACAAGAGCTGCGAGAAGACTCGAGTCTTCTCGCAGCTCTTGTCCGTTTTT	CDS	NM_002801.x-298s1c1	2
TRCN0000003937	PSMB10	CCGGATACGCGAGCCACTAACGATTCTCGAGAATCGTTAGTGGCTCGCGTATTTTTT	CDS	NM_002801.x-268s1c1	3
TRCN0000010833	PSMB10	CCGGTGCAGGCTATGGAGGTGGAGTCTCGAGACTCCACCTCCATAGCCTGCATTTTT	CDS	NM_002801.x-901s1c1	4
TRCN0000151756	RCBTB2	CCGGCCTGAAGAACTTTATCAGCAACTCGAGTTGCTGATAAAGTTCTTCAGGTTTTTTG	CDS	NM_001268.2-1976s1c1	1
TRCN0000151907	RCBTB2	CCGGCCCTGGCAATTAAATTGTCAACTCGAGTTGACAATTTAATTGCCAGGGTTTTTTG	3UTR	NM_001268.2-2639s1c1	2
TRCN0000152592	RCBTB2	CCGGGTGAAGTATGATGCACAGGATCTCGAGATCCTGTGCATCATACTTCACTTTTTTG	CDS	NM_001268.2-1869s1c1	3
TRCN0000154627	RCBTB2	CCGGCACAAACTGCTGTGGCTGTTTCTCGAGAAACAGCCACAGCAGTTTGTGTTTTTTG	CDS	NM_001268.2-584s1c1	4
TRCN0000155938	RCBTB2	CCGGCCCTGGTCTCAGTATATGCTACTCGAGTAGCATATACTGAGACCAGGGTTTTTTG	3UTR	NM_001268.2-2314s1c1	5
TRCN0000014568	TAF9	CCGGCCTTGCTGAATGTAACATGTACTCGAGTACATGTTACATTCAGCAAGGTTTTT	3UTR	NM_003187.3-988s1c1	1
TRCN0000014569	TAF9	CCGGCCTTCCGATATGTGACCACAACTCGAGTTGTGGTCACATATCGGAAGGTTTTT	CDS	NM_003187.3-315s1c1	2
TRCN0000014570	TAF9	CCGGCCATCATTAATCGGGTCCAAACTCGAGTTTGGACCCGATTAATGATGGTTTTT	CDS	NM_003187.3-833s1c1	3
TRCN0000014571	TAF9	CCGGCCCTCACAGGTCAAAGGTTTACTCGAGTAAACCTTTGACCTGTGAGGGTTTTT	CDS	NM_003187.3-729s1c1	4
TRCN0000014572	TAF9	CCGGGCCGAAAGATGCACAGATGATCTCGAGATCATCTGTGCATCTTTCGGCTTTTT	CDS	NM_003187.3-223s1c1	5
TRCN0000135528	TMEM19	CCGGCTTGATAACAACGCAGTGAATCTCGAGATTCACTGCGTTGTTATCAAGTTTTTTG	CDS	NM_018279.2-1505s1c1	1
TRCN0000136115	TMEM19	CCGGGCTGGATTACTAGGATCAATTCTCGAGAATTGATCCTAGTAATCCAGCTTTTTTG	CDS	NM_018279.2-1373s1c1	2
TRCN0000136586	TMEM19	CCGGCCCTACTATGGTGATGATCTACTCGAGTAGATCATCACCATAGTAGGGTTTTTTG	3UTR	NM_018279.2-2765s1c1	3
TRCN0000137230	TMEM19	CCGGGATCGTCTCTAATGGCCTTAACTCGAGTTAAGGCCATTAGAGACGATCTTTTTTG	CDS	NM_018279.2-772s1c1	4
TRCN0000137271	TMEM19	CCGGCCCTTAACCTTTAAGCCAGTTCTCGAGAACTGGCTTAAAGGTTAAGGGTTTTTTG	3UTR	NM_018279.2-2825s1c1	5
TRCN0000022324	EGLN2	CCGGCGCATGGCAGACAGCTTAAATCTCGAGATTTAAGCTGTCTGCCATGCGTTTTT	3UTR	NM_080732.1-1498s1c1	1
TRCN0000022325	EGLN2	CCGGGCTGCATCACCTGTATCTATTCTCGAGAATAGATACAGGTGATGCAGCTTTTT	CDS	NM_080732.1-1174s1c1	2
TRCN0000022326	EGLN2	CCGGGCCACTCTTTGACCGGTTGCTCTCGAGAGCAACCGGTCAAAGAGTGGCTTTTT	CDS	NM_080732.1-1280s1c1	3

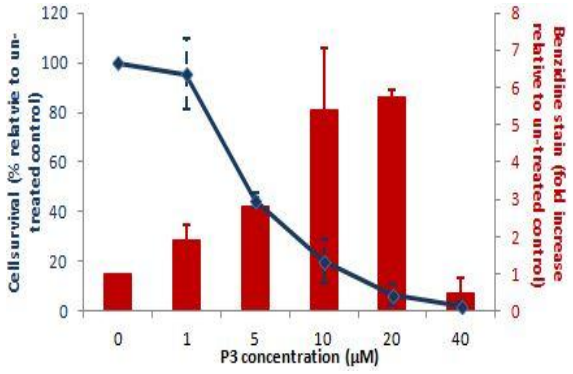
TRCN0000022327	EGLN2	CCGGACTGGGACGTTAAGGTGCATGCTCGAGCATGCACCTTAACGTCCCAGTTTTTT	CDS	NM_080732.1-1207s1c1	4
TRCN0000022328	EGLN2	CCGGCTGGGACGTTAAGGTGCATGGCTCGAGCCATGCACCTTAACGTCCCAGTTTTT	UNK	NM_080732.1-1208s1c1	5
TRCN0000000504	CHUK	CCGGGCATCATAAGGAGTTGGTGTACTCGAGTACACCAACTCCTTATGATGCTTTTT	3UTR	NM_001278.x-2866s1c1	1
TRCN0000000505	CHUK	CCGGCCAGATTATGAAGAAGTTGAACTCGAGTTCAACTTCTTCATAATCTGGTTTTT	CDS	NM_001278.x-281s1c1	2
TRCN0000000506	CHUK	CCGGCCAGCCTCTCAATGTGTTCTACTCGAGTAGAACACATTGAGAGGCTGGTTTTT	CDS	NM_001278.x-1194s1c1	3
TRCN0000000507	CHUK	CCGGGCAAATGAGGAACAGGGCAATCTCGAGATTGCCCTGTTCCTCATTTGCTTTTT	CDS	NM_001278.x-2274s1c1	4
TRCN0000000508	CHUK	CCGGGCGTGCCATTGATCTATATAACTCGAGTTATATAGATCAATGGCACGCTTTTT	CDS	NM_001278.x-1781s1c1	5
TRCN0000013338	BAZ1B	CCGGCCCACAACAAATCTAGCTCTACTCGAGTAGAGCTAGATTTGTTGTGGGTTTTT	3UTR	NM_023005.2-5516s1c1	1
TRCN0000013339	BAZ1B	CCGGGCCCTCTATGAAGTACCAGATCTCGAGATCTGGTACTTCATAGAGGGCTTTTT	CDS	NM_023005.2-4001s1c1	2
TRCN0000013340	BAZ1B	CCGGCGATACTTTATACGGCATAATCTCGAGATTATGCCGTATAAAGTATCGTTTTT	CDS	NM_023005.2-1112s1c1	3
TRCN0000013341	BAZ1B	CCGGGCAGATGACTTTGTTGGATATCTCGAGATATCCAACAAAGTCATCTGCTTTTT	CDS	NM_023005.2-1660s1c1	4
TRCN0000013342	BAZ1B	CCGGCGGGAAATCCAGGAAAGAGAACTCGAGTTCTCTTTCCTGGATTTCCCGTTTTT	CDS	NM_023005.2-2918s1c1	5
TRCN0000059073	PYCARD	CCGGGCCCACCAACCCAAGCAAGATCTCGAGATCTTGCTTGGGTTGGTGGGCTTTTTG	CDS	NM_013258.3-696s1c1	1
TRCN0000059074	PYCARD	CCGGCCTGCACTTTATAGACCAGCACTCGAGTGCTGGTCTATAAAGTGCAGGTTTTTG	CDS	NM_013258.3-573s1c1	2
TRCN0000059075	PYCARD	CCGGCGGAAGCTCTTCAGTTTCACACTCGAGTGTGAAACTGAAGAGCTTCCGTTTTTG	CDS	NM_013258.3-718s1c1	3
TRCN0000059076	PYCARD	CCGGGCTCAAGAAGTTCAAGCTGAACTCGAGTTCAGCTTGAACTTCTTGAGCTTTTTG	CDS	NM_013258.3-297s1c1	4
TRCN0000059077	PYCARD	CCGGCCTAAGGGAGTCCCAGTCCTACTCGAGTAGGACTGGGACTCCCTTAGGTTTTTG	CDS	NM_013258.3-780s1c1	5
TRCN0000020349	HTATIP2	CCGGCCCATCACCAAATCGGTAATTCTCGAGAATTACCGATTTGGTGATGGGTTTTT	3UTR	NM_006410.3-880s1c1	1
TRCN0000020350	HTATIP2	CCGGGCAGAATAAATCCGTCTTTATCTCGAGATAAAGACGGATTTATTCTGCTTTTT	CDS	NM_006410.3-160s1c1	2
TRCN0000020351	HTATIP2	CCGGGCAAACATTTCAACTTGCTATCTCGAGATAGCAAGTTGAAATGTTTGCTTTTT	CDS	NM_006410.3-483s1c1	3
TRCN0000020352	HTATIP2	CCGGCCTCTAAAGGAGCTGATAAATCTCGAGATTTATCAGCTCCTTTAGAGGTTTTT	CDS	NM_006410.3-504s1c1	4
TRCN0000020353	HTATIP2	CCGGCGTGGTTAGAGCAATGCTGAACTCGAGTTCAGCATTGCTCTAACCACGTTTTT	CDS	NM_006410.3-727s1c1	5
TRCN0000074173	HEXIM1	CCGGCCCTCCTTAAACGGAGCTATACTCGAGTATAGCTCCGTTTAAGGAGGGTTTTTG	3UTR	NM_006460.1-2067s1c1	1
TRCN0000074174	HEXIM1	CCGGGCATTGGAAACCGTACTACAACTCGAGTTGTAGTACGGTTTCCAATGCTTTTTG	CDS	NM_006460.1-1175s1c1	2

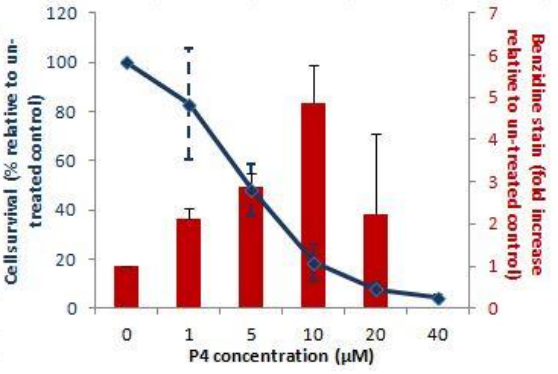
TRCN0000074175	HEXIM1	CCGGGCCCTATAACACCACGCAGTTCTCGAGAACTGCGTGGTGTTATAGGGCTTTTTG	CDS	NM_006460.1-1292s1c1	3
TRCN0000074176	HEXIM1	CCGGGCGGCATTGGAAACCGTACTACTCGAGTAGTACGGTTTCCAATGCCGCTTTTTG	CDS	NM_006460.1-1172s1c1	4
TRCN0000074177	HEXIM1	CCGGGCGGGACTTCTCGGAGACGTACTCGAGTACGTCTCCGAGAAGTCCCGCTTTTTG	CDS	NM_006460.1-1481s1c1	5
TRCN0000047188	ARHGAP4	CCGGGCCAAGTTCATGGAGCACAAACTCGAGTTTGTGCTCCATGAACTTGGCTTTTTG	CDS	NM_001666.2-730s1c1	1
TRCN0000047189	ARHGAP4	CCGGCGCTGCTGTCAGTAACTACTACTCGAGTAGTAGTTACTGACAGCAGCGTTTTTG	CDS	NM_001666.2-804s1c1	2
TRCN0000047190	ARHGAP4	CCGGCGAAACCTTCTACCTCACGAACTCGAGTTCGTGAGGTAGAAGGTTTCGTTTTTG	CDS	NM_001666.2-1350s1c1	3
TRCN0000047191	ARHGAP4	CCGGGAACATGGACTCTGTGTTTAACTCGAGTTAAACACAGAGTCCATGTTCTTTTTG	CDS	NM_001666.2-2661s1c1	4
TRCN0000047192	ARHGAP4	CCGGCATTCGCTTCATCAACCTCAACTCGAGTTGAGGTTGATGAAGCGAATGTTTTTG	CDS	NM_001666.2-1623s1c1	5
TRCN0000021794	CHD5	CCGGCGAATCCTGAACCATAGCTTTCTCGAGAAAGCTATGGTTCAGGATTCGTTTTT	CDS	NM_015557.1-1886s1c1	1
TRCN0000021795	CHD5	CCGGCCTGGAGATGAAGAACAAGTTCTCGAGAACTTGTTCTTCATCTCCAGGTTTTT	CDS	NM_015557.1-5443s1c1	2
TRCN0000021796	CHD5	CCGGCGTGTTCCTTTACTCCCTCTACTCGAGTAGAGGGAGTAAAGGAACACGTTTTT	CDS	NM_015557.1-2308s1c1	3
TRCN0000021797	CHD5	CCGGGCTACAGAACATGAACGAGTACTCGAGTACTCGTTCATGTTCTGTAGCTTTTT	CDS	NM_015557.1-3931s1c1	4
TRCN0000021798	CHD5	CCGGCGCAAGAAGAAGAGGATTGATCTCGAGATCAATCCTCTTCTTCTTGCGTTTTT	CDS	NM_015557.1-1079s1c1	5
TRCN0000053773	S100A8	CCGGCCACAAGTACTCCCTGATAAACTCGAGTTTATCAGGGAGTACTTGTGGTTTTTG	CDS	NM_002964.3-103s1c1	1
TRCN0000053774	S100A8	CCGGGTCTGGTTCAAAGAGTTGGATCTCGAGATCCAACTCTTTGAACCAGACTTTTTG	CDS	NM_002964.3-212s1c1	2
TRCN0000053775	S100A8	CCGGTCAACACTGATGGTGCAGTTACTCGAGTAACTGCACCATCAGTGTTGATTTTTG	CDS	NM_002964.3-234s1c1	3
TRCN0000053776	S100A8	CCGGGAACTCTATCATCGACGTCTACTCGAGTAGACGTCGATGATAGAGTTCTTTTTG	CDS	NM_002964.3-82s1c1	4

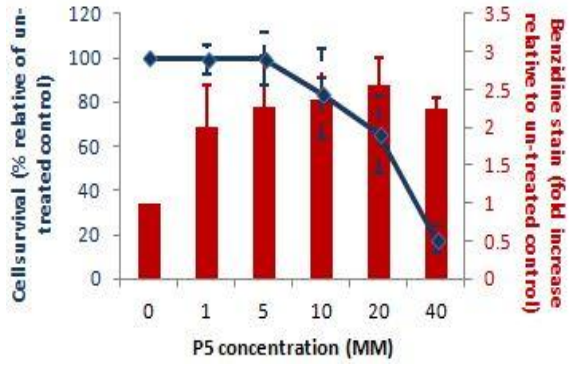
APPENDIX III

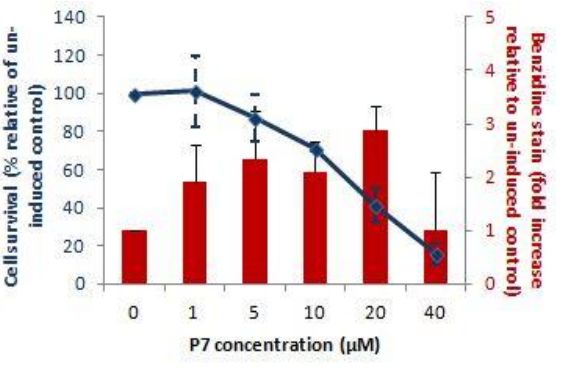

Cellsurvival (% relative to un-treated control)
Benzidine stain (fold increase relative to un-treated control)
P1 concentratrion (µM)

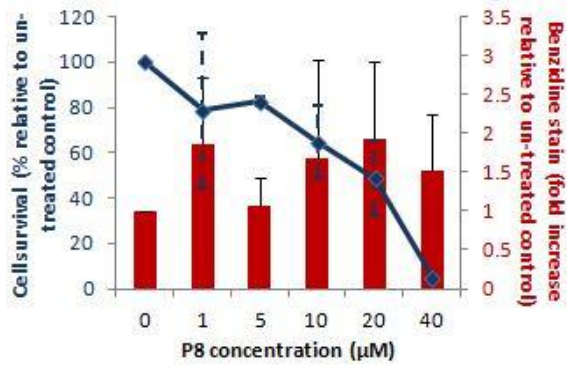

Cellsurvival (% relative to un-treated control)
Benzidine stain (fold increase relative to un-treated control)
P2 concentration (µM)

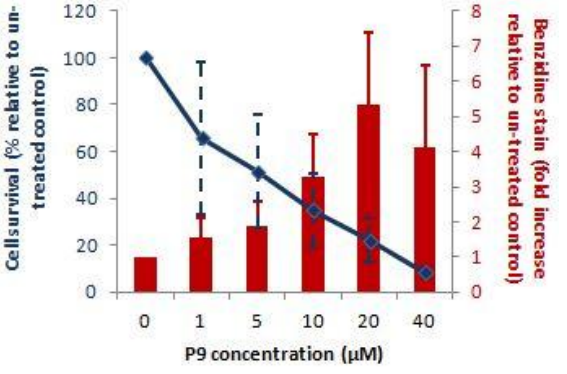

Cellsurvival (% relative to un-treated control)
Benzidine stain (fold increase relative to un-treated control)
P3 concentration (µM)


Cellsurvival (% relative to un-treated control)
Benzidine stain (fold increase relative to un-treated control)
P4 concentration (µM)


Cellsurvival (% relative of un-treated control)
Benzidine stain (fold increase relative to un-treated control)
P5 concentration (MM)


Cellsurvival (% relative of un-induced control)
Benzidine stain (fold increase relative to un-induced control)
P7 concentration (µM)


Cellsurvival (% relative to un-treated control)
Benzidine stain (fold increase relative to un-treated control)
P8 concentration (µM)


Cellsurvival (% relative to un-treated control)
Benzidine stain (fold increase relative to un-treated control)
P9 concentration (µM)

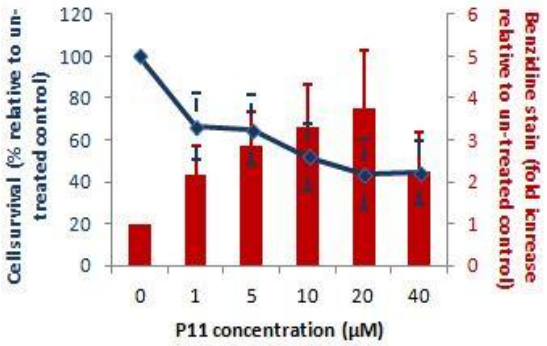


Figure III.I, Investigation of the haemoglobin inducing ability (Benzidine stain) and cytotoxicity (Trypan blue) of resveratrol and nine hydroxystilbenic derivatives in K562 cell line. Concentration curves were prepared for each compound in order to determine the concentration with the best HbF inducing activity while having a moderate cytotoxicity. The results are the average of 3 experiments for each agent and error bars correspond to the standard deviation.

APPENDIX IV

Table IV.I, List of proteins that are significantly differentially expressed for each of the four ratios after sorting based on their Log_2 values and selection of proteins with a p-value<0.05. Protein ID refers to the Uniprot Assession number corresponding to each protein. Protein description lists the full name of the protein. Protein name refers to the acronym of the protein which usually corresponds to the gene name. Average ratio corresponds to the average of the differential expression of the protein from three experiments. Log_2 value is the log_2 value of the average ratio and the p-value states the statistical significance of the log_2 value of each protein. The coverage is the percentage of the protein sequence that was identified through peptides and MW corresponds to the molecular weight of each protein.

Differentially expressed protein in treated over un-treated of healthy cultures (ratio 1)

Protein ID	*Protein Description*	*Protein Name*	*Average ratio*	*Log2 value*	*P-value*	*Coverage*	*MW (kDa)*
P52564	Dual specificity mitogen-activated protein kinase kinase 6	MAP2K6	2.488	1.271828	0	7.49	37.5
Q14738	Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit delta isoform	PPP2R5D	2.115	1.037499	5.64E-14	4.15	69.9
Q15006	ER membrane protein complex subunit 2	EMC2	1.892	0.876753	2.13E-10	6.4	34.8
P51784	Ubiquitin carboxyl-terminal hydrolase 11	USP11	1.652	0.681055	8.06E-07	1.66	109.7
Q92734	Protein TFG	TFG	1.642	0.672295	1.11E-06	6.5	43.4
Q14137	Ribosome biogenesis protein BOP1	BOP1	1.526	0.566596	4.05E-05	3.08	83.6
P42785	Lysosomal Pro-X carboxypeptidase	PRCP	1.51	0.55139	6.48E-05	5.24	55.8
Q9UDX4	SEC14-like protein 3	SEC14L3	1.468	0.510693	0.000216	5	46
Q15363	Transmembrane emp24 domain-containing protein 2	TMED2	1.462	0.504785	0.000255	7.96	22.7
Q494V2	Coiled-coil domain-containing protein 37	CCDC37	1.409	0.451513	0.001072	1.15	71.1
O60888	Protein CutA	CUTA	1.371	0.41207	0.002834	7.82	19.1
Q96EY1	DnaJ homolog subfamily A member 3, mitochondrial	DNAJA3	1.367	0.407855	0.00313	3.33	52.5
Q9HBI1	Beta-parvin	PARVB	1.365	0.405742	0.003289	3.85	41.7
Q13190	Syntaxin-5	STX5	1.361	0.401508	0.003629	3.38	39.6

Q9Y5Y2	Cytosolic Fe-S cluster assembly factor NUBP2	NUBP2	1.357	0.397262	0.004003	9.96	28.8
Q00341	Vigilin	HDLBP	1.343	0.382301	0.005613	1.42	141.4
O95236	Apolipoprotein L3	APOL3	1.328	0.366096	0.007997	2.74	44.3
Q9NP58	ATP-binding cassette sub-family B member 6, mitochondrial	ABCB6	1.323	0.360654	0.008982	4.99	93.8
Q8TEQ6	Gem-associated protein 5	GEMIN5	1.309	0.345306	0.012365	0.93	168.5
Q86TX2	Acyl-coenzyme A thioesterase 1	ACOT1	1.308	0.344204	0.012646	4.75	46.2
P52888	Thimet oligopeptidase	THOP1	1.307	0.3431	0.012934	2.9	78.8
P61201	COP9 signalosome complex subunit 2	COPS2	1.3	0.335353	0.015121	7.9	51.6
Q8N183	Mimitin, mitochondrial	NDUFAF2	1.298	0.333132	0.015806	10.65	19.8
P11216	Glycogen phosphorylase, brain form	PYGB	1.297	0.33202	0.016159	0.25	632.4
Q9NU22	Midasin	MDN1	1.297	0.33202	0.016159	1.54	96.6
P37198	Nuclear pore glycoprotein p62	NUP62	1.293	0.327564	0.017643	5.36	53.2
Q9ULZ3	Apoptosis-associated speck-like protein containing a CARD	PYCARD	1.289	0.323094	0.01925	8.21	28.9
P40306	Proteasome subunit beta type-10	PSMB10	1.289	0.323094	0.01925	7.33	21.6
O60613	15 kDa selenoprotein	SEP15	1.281	0.314112	0.022871	8.02	17.8
O43264	Centromere/kinetochore protein zw10 homolog	ZW10	1.28	0.312985	0.023365	1.16	88.8
Q9BRT2	Mitochondrial nucleoid factor 1	UQCC2	1.279	0.311858	0.023868	9.52	14.9
O15145	Actin-related protein 2/3 complex subunit 3	ARPC3	1.276	0.30847	0.025437	15.17	20.5
P17612	cAMP-dependent protein kinase catalytic subunit alpha	PRKACA	1.268	0.299396	0.030085	2.56	58.2
Q9NP81	Serine--tRNA ligase, mitochondrial	SARS2	1.268	0.299396	0.030085	2.7	40.6
Q9Y3A6	Transmembrane emp24 domain-containing protein 5	TMED5	1.266	0.297119	0.03136	5.24	26
Q9BUP3	Oxidoreductase HTATIP2	HTAI2	1.262	0.292553	0.034057	4.13	27
Q9Y6M1	Insulin-like growth factor 2 mRNA-binding protein 2	IF2B2	1.261	0.29141	0.034763	4.84	66.1
P02647	Apolipoprotein A-I	APOA1	1.259	0.28912	0.036214	5.99	30.8

P60983	Glia maturation factor beta	GMFB	1.257	0.286826	0.037718	22.54	36.5
Q7L5D6	Golgi to ER traffic protein 4 homolog	GET4	1.257	0.286826	0.037718	4.59	16.7
P19784	Casein kinase II subunit alpha'	CSNK2A2	1.255	0.284529	0.039278	3.71	41.2
P51157	Ras-related protein Rab-28	RAB28	1.254	0.283379	0.040079	10.86	24.8
Q96B26	Exosome complex component RRP43	EXOSC8	1.251	0.279923	0.042571	4.71	30
P10243	Myb-related protein A	MYBL1	1.248	0.276459	0.045198	10.83	60.3
Q8WVJ2	NudC domain-containing protein 2	NUDCD2	1.248	0.276459	0.045198	2.79	17.7
O95199	RCC1 and BTB domain-containing protein 2	RCBTB2	1.248	0.276459	0.045198	1.45	85.8
Q7Z5R6	Amyloid beta A4 precursor protein-binding family B member 1-interacting protein	APBB1IP	1.244	0.271828	0.048924	1.35	73.1
O75688	Protein phosphatase 1B	PPM1B	0.854	-0.27085	0.049742	3.76	52.6
Q9NUB1	Acetyl-coenzyme A synthase 2-like, mitochondrial	ACSS1	0.851	-0.27593	0.045613	2.32	74.8
O95630	STAM-binding protein	STAMBP	0.851	-0.27593	0.045613	4.01	48
P03928	ATP synthase protein 8	ATP8	0.848	-0.28102	0.041764	23.36	11.7
P17096	High mobility group protein HMG-I/HMG-Y O	HMGA1	0.848	-0.28102	0.041764	13.24	8
P16333	Cytoplasmic protein NCK1	NCK1	0.847	-0.28272	0.040541	3.71	42.8
Q6B0K9	Haemoglobin subunit mu	HBM	0.845	-0.28614	0.038181	45.39	15.6
O43772	Mitochondrial carnitine/acylcarnitine carrier protein	SLC25A20	0.841	-0.29298	0.033796	6.98	32.9
Q01658	Protein Dr1	DR1	0.841	-0.29298	0.033796	7.39	19.4
Q9BVJ6	U3 small nucleolar RNA-associated protein 14 homolog A	UTP14A	0.84	-0.2947	0.032767	1.56	87.9
Q9UFF9	CCR4-NOT transcription complex subunit 8	CNOT8	0.839	-0.29642	0.031763	2.19	40.8
Q9Y371	Endophilin-B1	SH3GLB1	0.839	-0.29642	0.031763	3.24	48.5
Q15645	Pachytene checkpoint protein 2 homolog	TRIP13	0.839	-0.29642	0.031763	4.79	33.5
Q00169	Phosphatidylinositol transfer protein alpha isoform	PITPNA	0.836	-0.30158	0.028902	9.63	31.8
P29590	Protein PML	PML	0.836	-0.30158	0.028902	1.36	97.5

Q9UP52	Transferrin receptor protein 2	TFR2	0.834	-0.30504	0.027115	2.75	88.7
O75563	Src kinase-associated phosphoprotein 2	SKAP2	0.828	-0.31546	0.022294	2.79	41.2
Q99496	E3 ubiquitin-protein ligase RING2	RNF2	0.825	-0.32069	0.020166	4.17	37.6
O75380	NADH dehydrogenase (ubiquinone) iron-sulfur protein 6, mitochondrial	NDUFS6	0.824	-0.32244	0.019495	17.74	13.7
Q99543	DnaJ homolog subfamily C member 2	DNAJC2	0.822	-0.32595	0.01821	2.74	72
P61011	Signal recognition particle 54 kDa protein	SRP54	0.817	-0.33475	0.015304	2.78	55.7
P20339	Ras-related protein Rab-5A	RAB5A	0.813	-0.34183	0.013272	30.23	23.6
P05204	Non-histone chromosomal protein HMG-17	HMGN2	0.812	-0.34361	0.012801	24.44	9.4
Q9C0D2	Centrosomal protein KIAA1731	KIAA1731	0.811	-0.34538	0.012345	0.88	295
Q8TBG4	Ethanolamine-phosphate phospho-lyase	ETNPPL	0.807	-0.35252	0.010655	2.66	91.8
Q09161	Nuclear cap-binding protein subunit 1	NCBP1	0.807	-0.35252	0.010655	5.01	55.6
Q16594	Transcription initiation factor TFIID subunit 9	TAF9	0.805	-0.3561	0.009887	8.71	29
Q16777	Histone H2A type 2-C	HIST2H2AC	0.8	-0.36509	0.008172	62.79	14
P04264	Keratin, type II cytoskeletal 1	K2C1	0.8	-0.36509	0.008172	28.57	66
P49247	Ribose-5-phosphate isomerase	RPIA	0.797	-0.37051	0.007272	2.57	33.2
P02533	Keratin, type I cytoskeletal 14	K1C14	0.795	-0.37413	0.00672	15.89	51.5
P02538	Keratin, type II cytoskeletal 6A	K2C6A	0.792	-0.37959	0.005961	16.49	60
P49189	4-trimethylaminobutyraldehyde dehydrogenase	ALDH9A1	0.785	-0.39239	0.004473	2.43	53.8
Q9NVR0	Kelch-like protein 11	KLHL11	0.767	-0.42586	0.002035	0.78	223
P12883	Myosin-7	MYH7	0.767	-0.42586	0.002035	1.84	80.1
Q9UBQ0	Vacuolar protein sorting-associated protein 29	VPS29	0.767	-0.42586	0.002035	5.49	20.5
P98171	Rho GTPase-activating protein 4	ARHGAP4	0.766	-0.42774	0.001943	1.16	105
Q99436	Proteasome subunit beta type-7	PSMB7	0.762	-0.4353	0.001613	3.61	29.9
Q96KS0	Egl nine homolog 2	EGLN2	0.758	-0.44289	0.001334	2.21	43.6

Q9H9J2	39S ribosomal protein L44, mitochondrial	MRPL44	0.752	-0.45435	0.000996	3.92	37.5
P62328	Thymosin beta-4	TYB4	0.737	-0.48342	0.000462	45.45	5
P23786	Carnitine O-palmitoyltransferase 2, mitochondrial	CPT2	0.736	-0.48538	0.000438	1.82	73.7
P32455	Interferon-induced guanylate-binding protein 1	GBP1	0.736	-0.48538	0.000438	2.87	67.9
P13807	Glycogen (starch) synthase, muscle	GYS1	0.723	-0.51109	0.000213	1.9	83.7
P08779	Keratin, type I cytoskeletal 16	K1C16	0.707	-0.54338	8.27E-05	16.07	51.2
P13284	Gamma-interferon-inducible lysosomal thiol reductase	IF130	0.706	-0.54542	7.77E-05	4.4	27.9
O15111	Inhibitor of nuclear factor kappa-B kinase subunit alpha	CHUK	0.703	-0.55156	6.45E-05	1.21	84.6
A6NN90	Uncharacterised protein C2orf81	C2orf81	0.675	-0.6102	9.84E-06	2.75	63
O94992	Protein HEXIM1	HEXIM1	0.666	-0.62956	5.09E-06	3.9	40.6
Q15654	Thyroid receptor-interacting protein 6	TRIP6	0.66	-0.64262	3.23E-06	5.46	50.3
Q9UPN9	E3 ubiquitin-protein ligase TRIM33	TRIM33	0.654	-0.6558	2.03E-06	0.62	122.5
Q9NZZ3	Charged multivesicular body protein 5	CHMP5	0.648	-0.66909	1.25E-06	7.31	24.6
P13647	Keratin, type II cytoskeletal 5	KRT5	0.636	-0.69606	4.59E-07	15.42	62.3
Q8N960	Centrosomal protein of 120 kDa	CEP210	0.632	-0.70516	3.25E-07	0.91	112.6
Q8IUE6	Histone H2A type 2-B	HIST2H2AB	0.62	-0.73282	1.1E-07	56.92	14
Q93077	Histone H2A type 1-C	HIST1H2AC	0.589	-0.80682	5.07E-09	62.31	14.1
Q96HH6	Transmembrane protein 19	TMEM19	0.544	-0.92148	2.46E-11	11.01	36.4
Q14563	Semaphorin-3A	SEMA3A	0.514	-1.00332	3.63E-13	3.63	88.8

Differentially expressed proteins in the ratio of treated over un-treated in thalassaemic cultures (ratio 2)

Protein ID	*Protein description*	*Protein name*	*Average ratio*	*Log2 value*	*P-value*	*Coverage*	*MW (kDa)*
Q8IUE6	Histone H2A type 2-B OS=Homo sapiens	HIST2H2AB	3.018	1.74461743	0	56.92	14
A6NN90	Uncharacterised protein C2orf81	C2orf81	2.525	1.48730801	0	2.75	63
P52564	Dual specificity mitogen-activated protein kinase kinase 6	MAP2K6	2.309	1.3582928	0	7.49	37.5
Q7Z2X4	PTB-containing, cubilin and LRP1-interacting protein	PID1	2.131	1.24255522	0	2.8	28.3
Q96HH6	Transmembrane protein 19	TMEM19	2.099	1.22072679	0	11.01	36.4
Q93077	Histone H2A type 1-C	HIST1H2AC	1.588	0.81823554	5.9641E-09	62.31	14.1
Q8N3Y1	F-box/WD repeat-containing protein 8	FBXW8	1.505	0.74078811	1.386E-07	2.68	67.4
Q8N183	Mimitin, mitochondrial	NDUFAF2	1.427	0.66400996	2.3446E-06	10.65	19.8
P22492	Histone H1t	HIST1H1T	1.424	0.66097377	2.6066E-06	15.46	22
P62328	Thymosin beta-4	TMSB4X	1.414	0.65080674	3.7043E-06	45.45	5
Q96IU4	Alpha/beta hydrolase domain-containing protein 14B	ABHD14B	1.41	0.64671979	4.2603E-06	6.19	22.3
P05204	Non-histone chromosomal protein HMG-17	HMGN2	1.399	0.63542059	6.2449E-06	24.44	9.4
Q14558	Phosphoribosyl pyrophosphate synthase-associated protein 1	PRPSAP1	1.347	0.58077447	3.6374E-05	3.93	39.4
Q9Y6A5	Transforming acidic coiled-coil-containing protein 3	TACC3	1.331	0.56353519	6.1545E-05	2.74	90.3
O96008	Mitochondrial import receptor subunit TOM40 homolog	TOMM40	1.309	0.53948972	0.00012513	3.05	37.9
O15533	Tapasin	TAPBP	1.281	0.5082951	0.00030146	1.79	47.6
Q13351	Kruppel-like factor 1	KLF1	1.224	0.44262818	0.00164874	5.25	38.2
Q96RU3	Formin-binding protein 1	FNBP1	1.22	0.43790577	0.00184841	3.08	71.3

Q9H3H3	UPF0696 protein C11orf68	C11orf68	1.214	0.43079305	0.00219134	4.38	27.3
Q8TBG4	Ethanolamine-phosphate phospho-lyase	ETNPPL	1.195	0.40803524	0.00371756	5.01	55.6
Q9Y6G3	39S ribosomal protein L42, mitochondrial	MRPL42	1.187	0.39834456	0.00462171	10.56	16.7
Q92783	Signal transducing adapter molecule 1	STAM	1.179	0.38858834	0.00572879	7.96	59.1
O00479	High mobility group nucleosome-binding domain-containing protein 4	HMGN4	1.173	0.38122764	0.00671661	7.78	9.5
P24863	Cyclin-C	CCNC	1.166	0.37259241	0.00806872	3.53	33.2
Q96RT1	Protein LAP2	ERBB2IP	1.162	0.36763469	0.00895073	1.56	158.2
Q15413	Ryanodine receptor 3	RYR3	1.158	0.36265988	0.00992134	0.37	551.7
Q96IZ7	Serine/Arginine-related protein 53	RSRC1	1.152	0.35516534	0.01156089	2.99	38.7
Q8NHV1	GTPase IMAP family member 7	GIMAP7	1.148	0.35014727	0.01278896	4.33	34.5
O00507	Probable ubiquitin carboxyl-terminal hydrolase FAF-Y	USP9Y	1.145	0.34637222	0.01378755	0.39	290.9
P16402	Histone H1.3	HIST1H1D	1.14	0.34005845	0.0156121	45.25	22.3
Q9UJ70	N-acetyl-D-glucosamine kinase	NAGK	1.13	0.3273474	0.0199396	2.03	37.4
Q92896	Golgi apparatus protein 1	GLG1	1.13	0.3273474	0.0199396	1.1	134.5
Q10713	Mitochondrial-processing peptidase subunit alpha	PMPCA	1.12	0.31452336	0.025332	3.24	58.2
P98175	RNA-binding protein 10	RBM10	1.12	0.31452336	0.025332	1.51	103.5
Q02539	Histone H1.1	HIST1H1A	1.12	0.31452336	0.025332	26.51	21.8
Q9NRN7	L-aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase	AASDHPPT	1.119	0.31323466	0.02593798	8.41	35.8
P04439	HLA class I histocompatibility antigen, A-3 alpha chain	HLA-A	1.117	0.31065381	0.02718933	18.08	40.8
P61011	Signal recognition particle 54 kDa protein	SRP54	1.117	0.31065381	0.02718933	2.78	55.7

Q9Y263	Phospholipase A-2-activating protein	PLAA	1.116	0.30936165	0.02783518	1.13	87.1
P04004	Vitronectin	VTN	1.115	0.30806833	0.02849482	4.81	54.3
Q13769	THO complex subunit 5 homolog	THOC5	1.113	0.30547822	0.02985644	1.32	78.5
P53041	Serine/threonine-protein phosphatase 5	PPP5C	1.111	0.30288344	0.03127622	4.21	56.8
P12955	Xaa-Pro dipeptidase	PEPD	1.111	0.30288344	0.03127622	5.68	54.5
Q93050	V-type proton ATPase 116 kDa subunit a isoform 1	ATP6V0A1	1.107	0.29767985	0.03429871	1.79	96.4
Q14667	UPF0378 protein KIAA0100	KIAA0100	1.105	0.29507099	0.03590576	0.72	253.5
P62820	Ras-related protein Rab-1A	RAB1A	1.104	0.2937648	0.03673421	37.56	22.7
P60983	Glia maturation factor beta	GMFB	1.101	0.28983909	0.03932274	22.54	16.7
Q96BP3	Peptidylprolyl isomerase domain and WD repeat-containing protein 1	PPWD1	1.1	0.28852815	0.04022095	1.7	73.5
Q5VUJ6	Leucine-rich repeat and calponin homology domain-containing protein 2	LRCH2	1.099	0.28721601	0.04113734	1.57	84.5
Q9H845	Acyl-CoA dehydrogenase family member 9, mitochondrial	ACAD9	1.098	0.28590268	0.04207221	4.03	68.7
Q6ZRH9	Uncharacterised protein FLJ46347		1.097	0.28458815	0.04302588	2.71	53.6
Q6STE5	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily D member 3	SMARCD3	1.096	0.28327242	0.04399865	3.52	55
Q9H0D6	5'-3' exoribonuclease 2	XRN2	1.094	0.28063736	0.04600275	1.16	108.5
Q7LDG7	RAS guanyl-releasing protein 2	GRP2	1.093	0.27931802	0.04703472	1.97	69.2
Q9H2W6	39S ribosomal protein L46, mitochondrial	MRPL46	1.093	0.27931802	0.04703472	7.53	31.7
A6NF01	Putative nuclear envelope pore membrane protein POM 121B	POM121B	0.743	-0.2775413	0.0484552	3	83
P16989	Y-box-binding protein 3	YBX3	0.743	-0.2775413	0.0484552	7.53	40.1
P36959	GMP reductase 1	GMPR	0.738	-0.2872827	0.04109037	7.83	37.4

Q8N766	ER membrane protein complex subunit 1	EMC1	0.737	-0.2892389	0.03973186	1.71	111.7
Q9BZI7	Regulator of nonsense transcripts 3B	UPF3B	0.737	-0.2892389	0.03973186	3.11	57.7
Q5T619	Zinc finger protein 648	ZN648	0.736	-0.2911977	0.0384099	2.11	62.3
Q6NYC1	Bifunctional arginine demethylase and lysyl-hydroxylase JMJD6	JMJD6	0.735	-0.2931592	0.03712379	3.47	46.4
O75352	Mannose-P-dolichol utilization defect 1 protein	MPDU1	0.734	-0.2951234	0.03587285	4.05	26.6
P22033	Methylmalonyl-CoA mutase, mitochondrial	MUT	0.733	-0.2970903	0.03465641	2.27	83.1
Q6P1K2	Polyamine-modulated factor 1	PMF1	0.733	-0.2970903	0.03465641	5.85	23.3
Q8NBI5	Solute carrier family 43 member 3	SLC43A3	0.732	-0.2990598	0.03347378	1.22	54.5
Q9UPT8	Zinc finger CCCH domain-containing protein 4	ZC3H4	0.731	-0.3010321	0.03232431	2.23	140.2
Q9UDX4	SEC14-like protein 3	S14L3	0.73	-0.303007	0.03120732	5	46
Q8TCD5	5'(3')-deoxyribonucleotidase, cytosolic type	NT5C	0.729	-0.3049847	0.03012215	8.96	23.4
P41223	Protein BUD31 homolog	BUD31	0.727	-0.3089481	0.02804466	13.89	17
Q96MW1	Coiled-coil domain-containing protein 43	CCDC43	0.725	-0.3129225	0.02608665	5.8	25.2
Q00169	Phosphatidylinositol transfer protein alpha isoform	PITPN1	0.724	-0.3149138	0.02515085	9.63	31.8
Q567U6	Coiled-coil domain-containing protein 93	CCDC93	0.721	-0.3209042	0.0225088	2.06	73.2
Q96C36	Pyrroline-5-carboxylate reductase 2	PYCR2	0.72	-0.3229066	0.02168117	4.69	33.6
Q8IUZ5	5-phosphohydroxy-L-lysine phospho-lyase	PHYKPL	0.72	-0.3229066	0.02168117	2.44	49.7
Q9NP58	ATP-binding cassette sub-family B member 6, mitochondrial	ABCB6	0.718	-0.3269196	0.02010187	4.99	93.8
Q92542	Nicastrin	NCSTN	0.716	-0.3309439	0.01861993	1.69	78.4
O14602	Eukaryotic translation initiation factor 1A, Y-chromosomal	EIF1AY	0.713	-0.3370014	0.0165695	18.06	16.4

Q9BYN0	Sulfiredoxin-1	SRXN1	0.712	-0.3390262	0.01592978	10.95	14.3
P51784	Ubiquitin carboxyl-terminal hydrolase 11	USP11	0.706	-0.3512353	0.0125133	1.66	109.7
Q2TAY7	WD40 repeat-containing protein SMU1	SMU1	0.702	-0.3594324	0.01060015	3.51	57.5
P55795	Heterogeneous nuclear ribonucleoprotein H2	HNRNPH2	0.702	-0.3594324	0.01060015	17.37	49.2
P06401	Progesterone receptor	PGR	0.701	-0.361489	0.01016299	1.29	98.9
Q9UBW5	Bridging integrator 2	BIN2	0.7	-0.3635485	0.00974136	3.01	61.8
Q9BXJ9	N-alpha-acetyltransferase 15, NatA auxiliary subunit	NAA15	0.698	-0.3676764	0.00894296	1.73	101.2
Q9NZ63	Uncharacterised protein C9orf78	C9orf78	0.693	-0.3780481	0.00718876	4.84	33.7
P35236	Tyrosine-protein phosphatase non-receptor type 7	PTPN7	0.688	-0.3884949	0.00574046	2.22	40.5
P40306	Proteasome subunit beta type-10	PSMB10	0.687	-0.3905934	0.00548346	7.33	28.9
Q13492	Phosphatidylinositol-binding clathrin assembly protein	PICALM	0.686	-0.3926949	0.00523653	5.98	70.7
P53597	Succinyl-CoA ligase (ADP/GDP-forming) subunit alpha, mitochondrial	SUCLG1	0.686	-0.3926949	0.00523653	6.94	36.2
O95199	RCC1 and BTB domain-containing protein 2	RCBTB2	0.682	-0.4011317	0.00434316	1.45	60.3
Q9UIG0	Tyrosine-protein kinase BAZ1B	BAZ1B	0.656	-0.4572077	0.00115078	0.94	170.8
Q9NQW7	Xaa-Pro aminopeptidase 1	XPNPEP1	0.653	-0.4638205	0.00097437	2.89	69.9
Q8N806	Putative E3 ubiquitin-protein ligase UBR7	UBR7	0.633	-0.508698	0.00029814	3.06	48
Q96TA2	ATP-dependent zinc metalloprotease YME1L1	YM31L1	0.628	-0.5201389	0.00021709	1.42	86.4
P17066	Heat shock 70 kDa protein 6	HSPA6	0.627	-0.522438	0.00020352	13.69	71
Q92734	Protein TFG	TFG	0.625	-0.5270473	0.0001787	6.5	43.4
Q14738	Serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit delta isoform	PPP2R5D	0.615	-0.5503171	9.1222E-05	4.15	69.9
P09601	Haem oxygenase 1	HMOX1	0.61	-0.5620942	6.4269E-05	12.5	32.8

Q96B49	Mitochondrial import receptor subunit TOM6 homolog	TOMM6	0.603	-0.5787455	3.8725E-05	18.92	8
P62942	Peptidyl-prolyl cis-trans isomerase FKBP1A	FKBP1A	0.599	-0.5883475	2.874E-05	12.96	11.9
Q16594	Transcription initiation factor TFIID subunit 9	TAF9	0.598	-0.590758	2.6648E-05	8.71	29
Q96NH3	Protein broad-minded	TBC1D32	0.558	-0.6906383	9.0831E-07	2.55	144.7
Q8TDI0	Chromodomain-helicase-DNA-binding protein 5	CHDA	0.545	-0.7246472	2.5726E-07	2.71	222.9
P02765	Alpha-2-HS-glycoprotein	AHSG	0.541	-0.7352749	1.7145E-07	6.81	39.3
Q9UHY7	Enolase-phosphatase E1	ENOPH1	0.537	-0.7459814	1.1328E-07	4.6	28.9
Q9C0D2	Centrosomal protein KIAA1731	KIAA1731	0.486	-0.8899472	2.4894E-10	0.88	295
Q8N960	Centrosomal protein of 120 kDa	CEP120	0.464	-0.9567787	1.0259E-11	0.91	112.6
Q14563	Semaphorin-3A	SEMA3A	0.413	-1.1247617	1.2728E-15	3.63	88.8
Q92609	TBC1 domain family member 5	TBC1D5	0.35	-1.3635485	3.1662E-22	1.38	88.9

Differentially expressed proteins of thalassaemic un-treated / healthy un-treated samples (ratio 3)

Protein ID	*Protein description*	*Protein name*	*Average ratio*	*Log2 ratio*	*p-value*	*Coverage*	*MW (kDa)*
P52564	Dual specificity mitogen-activated protein kinase kinase 6	MAP2K6	6.203	2.566164	1.65E-08	7.49	32
Q494V2	Coiled-coil domain-containing protein 37	**CCDC37**	5.666	2.435528	8.43E-08	1.15	37.5
Q92734	Protein TFG	TFG	3.335	1.670884	0.000237	6.5	71.1
P02765	Alpha-2-HS-glycoprotein	AHSG	3.165	1.595403	0.000449	6.81	43.4
Q9H8M2	Bromodomain-containing protein 9	BRD9	3.044	1.539166	0.00071	1.17	39.3
P02787	Serotransferrin	TF	2.916	1.477188	0.001156	2.01	67
P02647	Apolipoprotein A-I	APOA1	2.596	1.309488	0.003969	5.99	77
Q7L5D6	Golgi to ER traffic protein 4 homolog	GET4	2.382	1.185371	0.009118	4.59	30.8
Q9NZD4	Alpha-haemoglobin-stabilizing protein	AHSP	2.331	1.154146	0.01112	35.29	36.5
Q9NU22	Midasin	MDN1	2.259	1.108882	0.014715	0.25	11.8
P02771	Alpha-fetoprotein	AFP	2.23	1.090241	0.01647	2.96	632.4
Q9Y5E8	Protocadherin beta-15	PCDHB15	2.21	1.077244	0.017801	3.18	68.6
Q15006	ER membrane protein complex subunit 2	EMC2	2.209	1.076591	0.01787	6.4	86.3
P30508	HLA class I histocompatibility antigen, Cw-12 alpha chain	HLA-C	2.173	1.052886	0.02055	13.93	34.8
Q9HBI1	Beta-parvin	PARVB	2.129	1.023373	0.024371	3.85	40.9
Q9NZJ9	Diphosphoinositol polyphosphate phosphohydrolase 2	NUDT4	2.118	1.0159	0.025432	13.33	41.7
P02774	Vitamin D-binding protein	VTDB	2.11	1.01044	0.026231	8.23	20.3
Q8N766	ER membrane protein complex subunit 1	EMC1	2.101	1.004274	0.02716	1.71	52.9
Q9BXD5	N-acetylneuraminate lyase	NPL	2.085	0.993245	0.028893	3.44	111.7
O15069	NAC-alpha domain-containing protein 1	NACAD	2.032	0.956098	0.035446	0.83	35.1
Q9UHY7	Enolase-phosphatase E1	ENOPH	2.022	0.94898	0.036836	4.6	161

Q14624	Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4	1.965	0.907727	0.045844	1.18	28.9
Q9UIG0	Tyrosine-protein kinase BAZ1B	BAZ1B	1.954	0.899628	0.047815	0.94	103.3
P42785	Lysosomal Pro-X carboxypeptidase	PRCP	1.95	0.896672	0.048552	5.24	170.8
Q10589	Bone marrow stromal antigen 2	BST2	0.564	-0.89304	0.049471	8.33	55.8
Q5RI15	Cytochrome c oxidase protein 20 homolog	COX20	0.564	-0.89304	0.049471	10.17	13.3
Q9NWV8	BRISC and BRCA1-A complex member 1	BABAM1	0.563	-0.8956	0.048823	3.34	36.5
P62820	Ras-related protein Rab-1A	RAB1A	0.562	-0.89816	0.04818	37.56	22.7
Q96BP3	Peptidylprolyl isomerase domain and WD repeat-containing protein 1	PPWD1	0.559	-0.90588	0.046287	1.7	73.5
P17900	Ganglioside GM2 activator	SAP3	0.559	-0.90588	0.046287	3.63	20.8
O75351	Vacuolar protein sorting-associated protein 4B	VPS4B	0.559	-0.90588	0.046287	3.6	49.3
P49821	NADH dehydrogenase (ubiquinone) flavoprotein 1, mitochondrial	NDUFV1	0.555	-0.91624	0.043846	7.76	50.8
O15533	Tapasin	TAPBP	0.554	-0.91884	0.043251	1.79	47.6
Q13155	Aminoacyl tRNA synthase complex-interacting multifunctional protein 2	AIMP2	0.553	-0.92145	0.042661	2.5	35.3
Q96RU3	Formin-binding protein 1	FNBP1	0.55	-0.9293	0.040926	3.08	71.3
Q92600	Cell differentiation protein RCD1 homolog	RQCD1	0.548	-0.93455	0.039798	3.01	33.6
O60684	Importin subunit alpha-7	KPNA6	0.544	-0.94512	0.037609	2.61	60
Q99426	Tubulin-folding cofactor B	TBCB	0.54	-0.95577	0.035508	4.51	27.3
P12724	Eosinophil cationic protein	RNASE3	0.537	-0.96381	0.03399	7.5	18.4
P27816	Microtubule-associated protein 4	MAP4	0.536	-0.9665	0.033494	1.13	120.9
O95155	Ubiquitin conjugation factor E4 B	UBE4B	0.529	-0.98546	0.030172	0.69	146.1
P17213	Bactericidal permeability-increasing protein	BPI	0.529	-0.98546	0.030172	4.93	53.9
Q9UFF9	CCR4-NOT transcription complex subunit 8	CNOT8	0.526	-0.99367	0.028825	4.79	33.5
Q96C01	Protein FAM136A	FAM136A	0.52	-1.01022	0.026264	7.25	15.6

Q8TBG4	Ethanolamine-phosphate phospho-lyase	ETNPPL	0.513	-1.02977	0.023494	5.01	55.6
P03928	ATP synthase protein 8	MT-ATP8	0.511	-1.03541	0.022745	13.24	8
Q7Z2X4	PTB-containing, cubilin and LRP1-interacting protein	PID1	0.501	-1.06392	0.019262	2.8	28.3
P13807	Glycogen (starch) synthase, muscle	GYS1	0.5	-1.0668	0.018937	1.9	83.7
P25774	Cathepsin S	CTSS	0.495	-1.0813	0.017376	3.32	37.5
P49863	Granzyme K	GZMK	0.487	-1.10481	0.015084	3.41	28.9
Q96I25	Splicing factor 45	RB<17	0.481	-1.12269	0.013522	5.49	44.9
P35499	Sodium channel protein type 4 subunit alpha	SCN4A	0.48	-1.1257	0.013275	0.54	207.9
Q9GZT3	SRA stem-loop-interacting RNA-binding protein, mitochondrial	SLIRP	0.478	-1.13172	0.01279	9.17	12.3
Q9UBQ0	Vacuolar protein sorting-associated protein 29	VPS29	0.477	-1.13474	0.012553	5.49	20.5
P06239	Tyrosine-protein kinase Lck	LCK	0.464	-1.17461	0.009769	3.54	58
Q07973	1,25-dihydroxyvitamin D(3) 24-hydroxylase, mitochondrial	CYP24A1	0.448	-1.22523	0.007033	1.36	58.8
P08779	Keratin, type I cytoskeletal 16	KRT16	0.441	-1.24795	0.006046	16.07	51.2
O43516	WAS/WASL-interacting protein family member 1	WIPF1	0.41	-1.35311	0.002915	1.59	51.2
Q93077	Histone H2A type 1-C	HIST1H2AC	0.402	-1.38154	0.002373	62.31	14.1
Q9BZR8	Apoptosis facilitator Bcl-2-like protein 14	BCL2L14	0.385	-1.44387	0.001492	1.83	36.6
Q86TC9	Myopalladin	MUPN	0.351	-1.57726	0.000521	2.12	145.2
P49189	4-trimethylaminobutyraldehyde dehydrogenase	ALDH9A1	0.349	-1.5855	0.000487	2.43	53.8
A6NN90	Uncharacterised protein C2orf81	C2orf81	0.346	-1.59796	0.000439	2.75	63
P62328	Thymosin beta-4	TMSB4X	0.298	-1.81342	6.63E-05	45.45	5
P05204	Non-histone chromosomal protein HMG-17	HMGN2	0.297	-1.81827	6.34E-05	24.44	9.4
Q8IUE6	Histone H2A type 2-B	HIST2H2AB	0.284	-1.88284	3.44E-05	56.92	14

Differentially expressed proteins in thalassaemic treated over healthy treated samples (ratio 4)

Protein ID	*Protein description*	*Protein name*	*Average ratio*	*Log2 value*	*P-value*	*Coverage*	*MW (kDa)*
P52564	Dual specificity mitogen-activated protein kinase kinase 6	MAP2K6	5.757	2.666704113	4.33209E-13	7.49	37.5
Q494V2	Coiled-coil domain-containing protein 37	CCDC37	3.652	2.010073666	4.73824E-08	1.15	71.1
Q9H8M2	Bromodomain-containing protein 9	BRD9	2.573	1.504838357	4.34608E-05	1.17	67
Q96HH6	Transmembrane protein 19	TMEM19	2.442	1.429450101	0.000102982	11.01	36.4
P02787	Serotransferrin	TF	2.334	1.364191461	0.000210422	2.01	77
Q9Y5E8	Protocadherin beta-15	PCDHB15	2.274	1.326619154	0.000313249	3.18	86.3
Q7LDG7	RAS guanyl-releasing protein 2	RASGRP2	2.061	1.184731405	0.001288105	1.97	69.2
P02771	Alpha-fetoprotein	AFP	1.842	1.022659962	0.005464255	2.96	68.6
Q9NZJ9	Diphosphoinositol polyphosphate phosphohydrolase 2	NUDT4	1.822	1.006909859	0.006228173	13.33	20.3
P04004	Vitronectin	VTN	1.795	0.985370744	0.007428374	4.81	54.3
Q9Y6G3	39S ribosomal protein L42, mitochondrial	MRPL42	1.777	0.970830582	0.008351912	10.56	16.7
O75380	NADH dehydrogenase (ubiquinone) iron-sulfur protein 6, mitochondrial	NDUSFS6	1.703	0.909465335	0.013481565	17.74	13.7
Q7L5D6	Golgi to ER traffic protein 4 homolog	GET4	1.693	0.900968873	0.014376915	4.59	36.5
P02647	Apolipoprotein A-I	APOA1	1.683	0.892422077	0.015330078	5.99	30.8
O95239	Chromosome-associated kinesin KIF4A	KIF4A	1.674	0.884686428	0.016240295	3.75	51.1
O43493	Trans-Golgi network integral membrane protein 2	TGOLN2	1.674	0.884686428	0.016240295	0.89	139.8
P02774	Vitamin D-binding protein	GC	1.67	0.881235003	0.016661503	8.23	52.9
P20701	Integrin alpha-L	ITGAL	1.662	0.874307282	0.017535995	1.62	128.7
O15514	DNA-directed RNA polymerase II subunit RPB4	POLR2D	1.654	0.867346135	0.018454978	10.56	16.3
Q9HBI1	Beta-parvin	PARVB	1.65	0.863852925	0.018931822	3.85	41.7
P55285	Cadherin-6	CDH6	1.644	0.858597199	0.019669556	1.77	88.3

P02765	Alpha-2-HS-glycoprotein	AHSG	1.634	0.849794884	0.020961424	6.81	39.3
Q9C0B1	Alpha-ketoglutarate-dependent dioxygenase FTO	FTO	1.63	0.846258865	0.021500845	3.17	58.2
Q5VTE6	Protein angel homolog 2	ANGEL2	1.628	0.8444876	0.021775567	2.57	62.3
Q9UBB4	Ataxin-10	ATXN10	1.606	0.824858793	0.025030383	3.16	53.5
P04114	Apolipoprotein B-100	APOB	1.605	0.823960198	0.025188973	0.61	515.3
Q9NPA8	Enhancer of yellow 2 transcription factor homolog	ENY2	1.603	0.822161326	0.025509064	16.83	11.5
Q9NZD4	Alpha-haemoglobin-stabilizing protein	AHSP	1.598	0.817654309	0.026326538	35.29	11.8
P02724	Glycophorin-A	GYPA	1.592	0.812227236	0.027340846	20.67	16.3
O15111	Inhibitor of nuclear factor kappa-B kinase subunit alpha	CHUK	1.588	0.808597813	0.02803784	1.21	84.6
A2RU67	Uncharacterised protein KIAA1467	KIAA1467	1.583	0.804048156	0.028933144	2.57	67
Q13564	NEDD8-activating enzyme E1 regulatory subunit	NAE1	1.582	0.8031365	0.029115474	2.81	60.2
P02775	Platelet basic protein	PPBP	1.579	0.800398071	0.029669114	18.75	13.9
Q06033	Inter-alpha-trypsin inhibitor heavy chain H3	ITIH3	1.577	0.79856956	0.030043813	2.47	99.8
Q96KS0	Egl nine homolog 2	EGLN2	1.576	0.797654435	0.030232863	2.21	43.6
Q96IZ7	Serine/Arginine-related protein 53	RSRC1	1.56	0.782932929	0.033417466	2.99	38.7
P23786	Carnitine O-palmitoyltransferase 2, mitochondrial	CPT2	1.551	0.774585587	0.03534759	1.82	73.7
Q15057	Arf-GAP with coiled-coil, ANK repeat and PH domain-containing protein 2	ACAP2	1.549	0.772724044	0.035790769	2.44	88
O14880	Microsomal glutathione S-transferase 3	MGST3	1.546	0.76992722	0.036465516	14.47	16.5
P02768	Serum albumin	ALB	1.524	0.749249803	0.041798284	9.2	69.3
Q9BXD5	N-acetylneuraminate lyase	NPL	1.522	0.747355259	0.042318342	3.44	35.1
Q8NF50	Dedicator of cytokinesis protein 8	DOCK8	1.514	0.739752106	0.044460824	0.86	238.4
Q93077	Histone H2A type 1-C	HIST1H2AC	1.513	0.738798888	0.044735778	62.31	14.1
Q9NU22	Midasin	MDN1	1.505	0.731150387	0.046994322	0.25	632.4
Q8IU89	Ceramide synthase 3	CERS3	1.504	0.730191467	0.047284137	5.22	46.3

Q15650	Activating signal cointegrator 1	TRIP4	1.499	0.725387284	0.048758833	1.38	66.1
Q6NZY4	Zinc finger CCHC domain-containing protein 8	ZCCHC8	1.497	0.723461122	0.049360838	1.98	78.5
P30508	HLA class I histocompatibility antigen, Cw-12 alpha chain	HLA-C	1.496	0.722497075	0.049664477	13.93	40.9
Q0VD83	Apolipoprotein B receptor	APOBR	1.495	0.721532385	0.049969885	1.38	114.8
Q9UFF9	CCR4-NOT transcription complex subunit 8	CNOT8	0.549	-0.723735045	0.049274847	4.79	33.5
O43427	Acidic fibroblast growth factor intracellular-binding protein	FIBP	0.547	-0.729000362	0.047646217	2.47	41.9
Q96BW5	Phosphotriesterase-related protein	PTER	0.545	-0.734284965	0.046057362	3.15	39
Q96C01	Protein FAM136A	FAM136A	0.545	-0.734284965	0.046057362	7.25	15.6
Q14258	E3 ubiquitin/ISG15 ligase TRIM25	TRIM	0.544	-0.736934543	0.045277691	3.33	70.9
Q96A26	Protein FAM162A	FAM162A	0.543	-0.739588997	0.044507771	7.14	17.3
P49006	MARCKS-related protein	MARCKSL1	0.542	-0.742248343	0.043747538	6.67	19.5
Q9UHL4	Dipeptidyl peptidase 2	DPP7	0.54	-0.747581787	0.042255872	8.13	54.3
Q14997	Proteasome activator complex subunit 4	PSME4	0.54	-0.747581787	0.042255872	0.71	211.2
Q9NYL4	Peptidyl-prolyl cis-trans isomerase FKBP11	FKBP11	0.538	-0.752935022	0.040802168	10.45	22.2
Q8TAE8	Growth arrest and DNA damage-inducible proteins-interacting protein 1	GADD45GIP1	0.536	-0.758308194	0.039385895	4.5	25.4
Q99614	Tetratricopeptide repeat protein 1	TTC1	0.535	-0.761002303	0.038691629	4.11	33.5
Q7L2H7	Eukaryotic translation initiation factor 3 subunit M	EIF3M	0.534	-0.763701453	0.038006519	4.28	42.5
P16989	Y-box-binding protein 3	YBX3	0.534	-0.763701453	0.038006519	4.73	18.8
P30536	Translocator protein	TSPO	0.534	-0.763701453	0.038006519	7.53	40.1
P03928	ATP synthase protein 8	MT-ATP8	0.534	-0.763701453	0.038006519	13.24	8
P61201	COP9 signalosome complex subunit 2	COPS2	0.533	-0.766405662	0.037330499	7.9	51.6
O95155	Ubiquitin conjugation factor E4 B	UBE4B	0.533	-0.766405662	0.037330499	0.69	146.1
Q10589	Bone marrow stromal antigen 2	BST2	0.53	-0.774548835	0.035356294	8.33	19.8
P27816	Microtubule-associated protein 4	MAP4	0.528	-0.780003265	0.034084354	1.13	120.9

Q9UHG3	Prenylcysteine oxidase 1	PCYOX1	0.526	-0.785478395	0.032847129	2.18	56.6
Q99426	Tubulin-folding cofactor B	TBCB	0.526	-0.785478395	0.032847129	4.51	27.3
P17900	Ganglioside GM2 activator	GM2A	0.518	-0.807589097	0.028234253	3.63	20.8
P49354	Protein farnesyltransferase/geranylgeranyltransferase type-1 subunit alpha	FNTA	0.516	-0.813170129	0.027162238	4.75	44.4
P05109	Protein S100-A8	S100AB	0.514	-0.818772835	0.026121578	56.99	10.8
Q15075	Early endosome antigen 1	EEA1	0.509	-0.832875538	0.023653388	0.85	162.4
P07766	T-cell surface glycoprotein CD3 epsilon chain	CD3E	0.509	-0.832875538	0.023653388	6.76	23.1
O60888	Protein CutA	CUTA	0.501	-0.855730591	0.020082422	7.82	19.1
Q96I25	Splicing factor 45	RBM17	0.5	-0.8586131	0.019667287	5.49	44.9
O75844	CAAX prenyl protease 1 homolog	ZMPSTE24	0.495	-0.873112669	0.017690792	2.11	54.8
P48595	Serpin B10	SERPINB10	0.488	-0.893660047	0.015188658	4.03	45.4
P04424	Argininosuccinate lyase	ASL	0.482	-0.911508048	0.013273796	2.59	51.6
P25774	Cathepsin S	CTSS	0.478	-0.923530576	0.012107353	3.32	37.5
Q07973	1,25-dihydroxyvitamin D(3) 24-hydroxylase, mitochondrial	CYP24A1	0.471	-0.944814135	0.01026353	1.36	58.8
Q9H2U1	Probable ATP-dependent RNA helicase DHX36	DHX36	0.469	-0.950953272	0.009780335	0.89	114.7
P12724	Eosinophil cationic protein	RNASE3	0.462	-0.972648343	0.008231093	7.5	18.4
O75351	Vacuolar protein sorting-associated protein 4B	VPS4B	0.461	-0.975774444	0.008026969	3.6	49.3
P49189	4-trimethylaminobutyraldehyde dehydrogenase	ALDH9A1	0.455	-0.994674649	0.006886566	2.43	53.8
P49863	Granzyme K	GZMK	0.449	-1.01382575	0.005881619	3.41	28.9
Q99447	Ethanolamine-phosphate cytidylyltransferase	PCYT2	0.442	-1.036494825	0.004864152	3.6	43.8
P17213	Bactericidal permeability-increasing protein	BPI	0.438	-1.049610325	0.004350982	4.93	53.9
Q92600	Cell differentiation protein RCD1 homolog	RQCD1	0.434	-1.062846152	0.003883349	3.01	33.6
P06239	Tyrosine-protein kinase Lck	LCK	0.433	-1.06617417	0.003773187	3.54	58
Q92609	TBC1 domain family member 5	TBC1D5	0.427	-1.086305125	0.003165241	1.38	88.9

P35499	Sodium channel protein type 4 subunit alpha	SCN4A	0.415	-1.127429858	0.002191719	0.54	207.9
Q86TC9	Myopalladin	MYPN	0.41	-1.144917285	0.001868014	2.12	145.2
O43516	WAS/WASL-interacting protein family member 1	WIPF1	0.387	-1.228207628	0.000847701	1.59	51.2
Q9GZT3	SRA stem-loop-interacting RNA-binding protein, mitochondrial	SLIRP	0.37	-1.293015924	0.000443414	9.17	12.3
Q9BZR8	Apoptosis facilitator Bcl-2-like protein 14	BCL1L14	0.364	-1.316602744	0.000347722	1.83	36.6

Differentially express proteins attributed to the disease state

Protein ID	*Protein name*	*Function*	*Ratio 4: Treated thal/healthy*	*Ratio 3: un-treated thal/healthy*	*Ratio 2: thal treated/un-treated*	*Ratio 1: healthy treated/un-treated*
Q9H8M2	Bromodomain-containing protein 9	possible role in chromatin remodelling and regulation of transcription	↑	↑		
P02787	Serotransferrin	responsible for transport of iron from sites of absorption and haem degradation, further roe in stimulating cell proliferation	↑	↑		
Q9Y5E8	Protocadherin beta-15	calcium-dependent cell -adhesion proteins	↑	↑		
P02771	Alpha-fetoprotein	binds copper, nickel and fatty acids and bilirubin	↑	↑		
Q9NZJ9	Diphosphoinositol polyphosphate phosphohydrolase 2	role in signal transduction	↑	↑		
P02774	Vitamin D-binding protein	carries vitamin D sterols and prevents polymerization of actin in plasma	↑	↑		
Q9NZD4	Alpha-haemoglobin-stabilizing protein	chaperone to prevent the harmful aggregation of alpha-haemoglobin during normal erythroid cell developments	↑	↑		
Q9BXD5	N-acetylneuraminate lyase	catalyzes cleavage of sialic acid to pyruvate via Schiff base intermediate	↑	↑		
P30508	HLA class I histocompatibility antigen, Cw-12 alpha chain	involved in presentation of foreign antigens to the immune system	↑	↑		
Q96C01	Protein FAM136A	mitochondrial protein	↓	↓		
O95155	Ubiquitin conjugation factor E4 B	catalyzes ubiquitin chain assembly	↓	↓		
Q10589	Bone marrow stromal antigen 2	IFN-induced antiviral host restriction factor	↓	↓		
P27816	Microtubule-associated protein 4	promotes microtubule assembly	↓	↓		
Q99426	Tubulin-folding cofactor B	involved in regulation of tubulin heterodimer dissociation	↓	↓		
P17900	Ganglioside GM2 activator	stimulates ganglioside GM2 degradation	↓	↓		

Q96I25	Splicing factor 45	involved in regulation of alternative splicing and utilization of cryptic splice sites	↓	↓		
P25774	Cathepsin S	thiol protease	↓	↓		
Q07973	1,25-dihydroxyvitamin D(3) 24-hydroxylase, mitochondrial	maintaining calcium homeostasis	↓	↓		
P12724	Eosinophil cationic protein	cytotoxin exhibiting antibacterial activity	↓	↓		
O75351	Vacuolar protein sorting-associated protein 4B	involved in endosomal multivesicular bodies pathway	↓	↓		
P49863	Granzyme K	serine-tupe endopeptidase activity	↓	↓		
P17213	Bactericidal permeability-increasing protein	antibacterial activity against Gram-negative bacterium	↓	↓		
Q92600	Cell differentiation protein RCD1 homolog	Component of CCR4-NOT complex, a cellular mRNA deadenylases	↓	↓		
P06239	Tyrosine-protein kinase Lck	important in selection and maturation of developing T-cells in thymus	↓	↓		
P35499	Sodium channel protein type 4 subunit alpha	mediated voltage-dependent sodium ion permeability of excitable membranes	↓	↓		
Q86TC9	Myopalladin	component of the sacromere	↓	↓		
O43516	WAS/WASL-interacting protein family member 1	plays a role in the reorganization of the actin cytoskeleton	↓	↓		
Q9GZT3	SRA stem-loop-interacting RNA-binding protein, mitochondrial	RNA binding protein that acts as a nuclear receptor corepressor	↓	↓		
Q9BZR8	Apoptosis facilitator Bcl-2-like protein 14	involved in apoptosis	↓	↓		
P02765	Alpha-2-HS-glycoprotein	promotes endocytosis, possesses opsonic properties and influences the mineral phase of bone	↑	↑	↓	
P52564	mitogen-activated protein kinase kinase 6	activation of MAP kinase signal transduction pathway in response to cytokines and all kinds of stresses	↑	↑	↑	↑
Q93077	Histone H2A type 1-C	component of nucleosome involved in transcription regulation and chromosomal stability	↑	↓	↑	↓

Q494V2	Coiled-coil domain-containing protein 37		↑	↑		↑
Q9HBI1	Beta-parvin	involved in integrin signalling via ILK and in activation of GTPases	↑	↑		↑
Q9NU22	Midasin	required for maturation and nuclear export of pre-60s ribosomal subunits	↑	↑		↑
P02647	Apolipoprotein A-I	participates in the reverse transport of cholesterol	↑	↑		↑
Q7L5D6	Golgi to ER traffic protein 4 homolog	component of the BAT3 complex involved in post-translational delivery of tail-anchored membrane proteins	↑	↑		↑
P03928	ATP synthase protein 8	mitochondrial membrane ATP synthase	↓	↓		↓
Q9UFF9	CCR4-NOT transcription complex subunit 8	Involved in mRNA degradation and translational repression during translational initiation and general transcriptional regulation	↓	↓		↓
P49189	4-trimethylaminobutyraldehyde dehydrogenase	catalyzes the irreversible oxidation of a broad range of aldehydes to corresponding acids	↓	↓		↓
Q92734	Protein TFG	Interacts with TANK &NEMO – proteins involved in the NF-κB pathway		↑	↓	↑
P05204	Non-histone chromosomal protein HMG-17	Involved in transcription regulation of gene containing or in close proximity to AT rich regions		↓	↑	↓
Q8TBG4	Ethanolamine-phosphate phospho-lyase	catalyzed breakdown of phosphoethanolamide		↓	↑	↓
P62328	Thymosin beta-4	involved in organization of cytoskeleton		↓	↑	↓
A6NN90	Uncharacterised protein C2orf81	uncharacterised		↓	↑	↓
Q8IUE6	Histone H2A type 2-B	core component of nucleosome		↓	↑	↓
Q15006	ER membrane protein complex subunit 2	endoplasmic reticulum protein		↑		↑
P42785	Lysosomal Pro-X carboxypeptidase	cleaves C-terminal amino acids linked to proline in peptide such as angiotensin II, III		↑		↑

Q9UBQ0	Vacuolar protein sorting-associated protein 29	component of retromer complex used in retrieval of lysosomal enzyme receptors	↓	↓
P13807	Glycogen (starch) synthase, muscle		↓	↓
P08779	Keratin, type I cytoskeletal 16		↓	↓
Q7Z2X4	PTB-containing, cubilin and LRP1-interacting protein	Over expression increase ROS production and significantly inhibit phosphorylation of IRS1 and serine phosphorylation of Akt which is restored by TFAM expression	↓	↑
O15533	Tapasin	involved in association of MHC class I transported	↓	↑
Q96RU3	Formin-binding protein 1	act as a link between RND2 singling and regulation of the actin cytoskeleton	↓	↑
P62820	Ras-related protein Rab-1A	regulated vesicular protein transport	↓	↑
Q96BP3	Peptidylprolyl isomerase domain and WD repeat-containing protein 1	accelerates folding of proteins	↓	↑
Q8N766	ER membrane protein complex subunit 1	ER membrane complex	↑	↓
Q9UIG0	Tyrosine-protein kinase BAZ1B	plays a central role in chromatin remodelling and acts as a transcription regulator, component of WICH complex	↑	↓
Q9UHY7	Enolase-phosphatase E1	bifunctional enzyme involved in amino-acid biosynthesis	↑	↓

LIST OF ABBREVIATIONS

2DE	Two Dimensional Electrophoresis
ABCB6	ATP-binding Cassette subunit-family B member 6
AFP	Alpha-haemoglobin stabilizing protein
AMG	Aorta-Gonad-Mesonephros
AP-1	Activator Protein 1
APC	Allphycocyanine
ARG1/2	Arginine 1/2
ARHGAP4	Rho GTPase-activating Protein 4
ATF-2	Activating Factor-2
BAZ1B	Bromodomain Adjacent to Zinc Finger Domain, 1B
BCL11A	B-cell Lymphoma/Leukaemia 11A
BFU-e	Burst Forming Unit, erythroid
BHQ	Black Hole Quencher
BP	Biological Process
BRE	TFIIB- recognition Element
BRG1	ATP-dependent helicase SMARCA4
BSA	Bovine Serum Albumin
bZIP	Basic Leucin Zipper domain
cAMP	cyclic Adenosine Monophosphate
CBP	CREB-binding Protein
CC	Cellular Component
CCR4	Chemokine (C-C motif) Receptor 4
CD235	Glycophorin A
CD71	Transferring receptor
Cdc2	Cell division cycle protein 2 homolog (CDK1, cyclin-dependent kinase 1)
cDNA	Complementary DNA
CEP120	Centrosomal protein 120kDa
CFU-e	Colony Forming Unit, erythroid
cGMP	cyclic Guanosine Monophospate
CHD4	Chromodomain-helicase DNA binding protein 4
CHD5	Chromodomain-helicase Binding protein 5
ChIP	Chromatin Immunoprecipitation
Chop	C/CBP homologous protein -10
CHUK	Conserved Helix-loop-helix Ubiquitous Kinase (IKKα)
CID	Collision-induced Dissociation
CML	Chronic Myeloid Leukaemia
COPS2	COP9 signalosome complex Subunit 2
CoREST	Corepressor for Element-1-silencing Transcription factor
COX	Cyclooxygenase
CP1	Cleft palate, isolated
CPT2	Carnitine O-palmitoyltransferase 2
CRE	Ca^{2+}/cAMP response element
CREB	Cyclic AMP Response Element Binding protein
CREBP	CREB-binding protein
CRISPR	Clustered Regularly Interspaced Short Palidromic Repeats
CUTA	Protein CutA
Da	Dalton
DBD	DNA-binding drug
Dec	Decitabine (5-aza-2'-deoxycytidine)
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DNAJA3	DNAJ Homolog Subfamily A Member 3
DNMT1	DNA Methyltransferase 1
dNTP	deoxyribonucleotide
DPE	Downstream Promoter Element
DR	Direct Repeat Elements
DR1	Down-Regulator of Transcription 1, TBP-Binding
DRED	Direct Repeat Erythroid-definitive

DSB	DNA double-strand breaks
EF	helix-loop-helix structural domain in calcium-binding proteins
EGLN2	Egl-9 Family Hypoxia-Inducible Factor 2
eIF2A	eukaryotic Translation Initiation Factor 2A
EKLF (KLF1)	Erythroid Kruppel-like Factor (Kruppel-like Factor 1)
EPC	Erythroid precursor cell
EPO	Erythropoietin
ER	Endoplasmic Reticulum
ERK	Extracellular signal-regulated kinase
ESI	Electrospray Ionisation
FBS	Foetal Bovine Serum
FCH	conserved region of ~60 amino acids homologous between FER and CIP4 proteins
FCS	Foetal Calf Serum
FOG-1	Friend of GATA1
FOXO	Forkhead box protein O
FOP	Fibroblast growth factor receptor 1 (FGFR1) Oncogene partner
GABA-A	γ-aminobutyric acid receptor A
GADD	Growth arrest- and DNA damage-inducible gene
GADD153	Growth arrest- and DNA damage-inducible gene 153
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GATA1	GATA-binding protein 1
GMFB	Glia Maturation Factor Beta
GPA	Glycophorin A
GYPA	Glycophorin A
H3	Histone 3
H3K27	Histone 3 Lysine 27
H3K36	Histone 3 Lysine 36
H3K4	Histone 3 Lysine 4
H3K4me2	Histone 3 Lysine 4 di-methylation
H3K9	Histone 3 Lysine 9
H4	Histone 4
H4R3	Histone 4 arginine 3
HAT	Histone Acetyltransferase
Hb	Haemoblogin
HbA	Adult haemoglobin
HBB	β-globin
HbF	Foetal haemoglobin
HBG	γ-globin
HCD	High-energy Collisional Dissociation
HDAC	Histone Deacetylase
HDR	Homology-directed repair
HEXIM1	Hemamethylene bis-acetamide-inducible protein 1
HIF	Hypoxia-induced Factor
HIST2H2AB/AC	Histone H2A type 2B /1C
HMG	High Mobility Group
HMOX1	Haem oxygenase
HPFH	Hereditary Persistence of Foetal Haemoglobin
HPLC	High Performance Liquid Chromatography
HRI	Haem-regulated inhibitor kinase
HS	Hypersensitive Sites
HSB1L	HSB1-like (S. cerevisiae)
HSC	Haematopoietic Stem Cell
HTATIP2	HIV-1 Tat Interactive Protein 2
HU	Hydroxyurea
IFN-γ	Interferon gamma
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta
IL-3	Interluekin-3
IL-6	Interluekin-6
Inr	Initiation element
IP	Immunoprecipitation
iPS	Induced Pluripotent Stem

ITGAL	Integrin Alpha-L
iTRAQ	Isobaric tags for Relative and Absolute Quantitation
JMJD6	Jumonji domain Containing 6
JNK	c-Jun N-terminal kinase
KEAP1	Kelch-like ECH-associated Protein 1
KIAA1731	Centrosomal protein KIAA1731
LB	Luria-Bertani
LBD1	LOB Domain-containing protein 1
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LCR	Locus Control Region
LMO2	LIM domain Only Protein 2
LSD1	Lysine-specific Histone Demethylase 1
MAP2K6	Dual specificity mitogen-activated protein kinase kinase 6
MAPK	Mitogen-activated protein kinase
MARE	Maf-response Element
MBD	Methyl cytosine-binding Domain
MBD2	Methyl cytosine-binding Domain 2
MBD3	Methyl cytosine-binding Domain 3
MCS	Multi-species Conserved Sequences
MDS	Myelodysplastic syndrome
MeCP2	Methyl CpG binding protein 2
MEKK	MAP Kinase Kinase Kinase
MEL	Mouse Erythroleukaemia
MF	Molecular Function
MKK	Dual specificity mitogen-activated protein Kinase Kinase
MRPL42	Mitochondrial 39S ribosomal protein L42
MS	Mass Spectrometry
MS/MS	Tandem Mass spectrometry
MW	Molecular weight
MYB	Myeloblastosis oncogene
MYBL1	Myb-Related Protein A
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCOR	Nuclear receptor co-repressor
NDUFAF2	NADH Dehydrogenase (Ubiquinone) Complex I, Assembly Factor 2
NDUFS6	NADH dehydrogenase (Ubiquinone) iron-sulfur protein 6
NF-E2	Nuclear Factor, Erythroid 2
NF-Y	Nuclear transcription factor Y
NF-κB	Nuclear Factor kappa-B
NHEJ	Non-homologous end joining
NO	Nitric oxide
NQO1	NADPH Quinine Oxyreductase 1
NRF2	Nuclear factor (erythroid-derived 2)-like 2
NTDT	Non-transfusion depended Thalassaemia
NuRD	Nucleosome Remodelling Deacetylase
PAGE	Polyacrylamide Gel Electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCAF	p300/CBP-associated Factor
PCBP2	Poly(rC)-binding protein 2
PCR	Polymerase Chain Reaction
PDE	Phophodiesterases
PE	Phycoerythrin
PHD1	Prolyl-4-hydroxylase 1
PI3K	Phosphatodylinositol-4,5-bisphosphate 3-kinase
PICALM	Phosphatidylinositol binding Clathrin Assembly protein
PITPNA	Phosphatidylinositol transfer protein Alpha isoform
PP2A	Protein phosphates 2
PPARγ	Peroxisome Proliferator-activated receptor gamma (γ)
PPP2R5D	Serine/threonin-protein phosphatise 2A Regulatory subunit delta
PPP5C	Serine/threonine-protein phosphatase 5
PRL-1	Phosphatase of Regenerating Liver protein-1

PRMT5	Protein Arginine Methyltransferase 5
PSMB10	Proteasome Subunit Beta type-10
P-TEFb	Positive Transcription Elongation Factor b
PTPN7	Protein Tyrosine Phosphatase, Non-Receptor Type 7
pVHL	von Hippel-Lidau tumor suppressor
PYCARD	Apoptosis-associated speck-like protein containing CARD
qRT-PCR	Quantitative real-time PCR
QTL	Quantitative Trait Locus
RASGRP2	RAS Guanyl-releasing protein 2
RBM10	RNA-binding protein 10
RCBTB2	RCC1 and BTB domain-containing Protein 2
RNA pol	RNA polymerase
RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive Oxygen Species
RSRC1	Serine/Arginine-related Protein 53
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RUNX1	Runt-related transcription factor 1
S100A8	S100 Calcium Binding Protein A8
SAR	Secretion-associated and RAS-related
SCF	Stem Cell Factor
SCFA	Short Chain Fatty Acids
SCL	Stem Cell Leukemia
SDS	Sodium Dodecyl Sulphate
SEC14L3	SEC14-like Protein 3 (S. Cerevisiae)
SEMA3A	Semaphorin-3A
SEPINB10	Serpin B10
sGC	soluble Guanyl Cyclise
shRNA	short hairpin RNA
siRNA	small interfering RNA
SIRT1	Silent mating type Information regulation 2 homolog 1
SMARCD3	SWI/SNF related matrix associated actin dependent regulator of chromatin subfamily D
SNP	Single Nucleotide Polymorphism
SOX6	SRY (sex determining region Y)-box 6
SP1	Specific Protein 1
SPF45	Splicing Factor 45
SRP54	Signal Recognition Particle 54kDa protein
SSE	Stage Selector Element
SWI/SNF	SWItch/Sucrose NonFermentable
TACC3	Transforming, Acidic Coiled-Coil Containing Protein
TAF	TBP-associated Factor
TAF9	TBP-associated Factor 9
Tal1	T-cell Acute lymphocytic leukemia 1
TALEN	Transcription Activator-like Effector Nucleases
TBC1D5	TBC1 Domain family member 5
TBP	TATA-binding Protein
TF	Serotransferin
THOC5	THO complex subunit 5 homolog
TMEM19	Transmembrane protein 19
TNF-α	Tumour-necrosis Factor α
TR2/4	Testicular Receptor 2/4
TRD	Transcriptional Repression Domain
TRIM28	Tripartite motif-containing 28
USP11	Ubiquitin Carboxyl-terminal hydrolase 11
UTR	Un-translated Region
VNT	Vitronectin
VTDB	Vitamin B Binding protein
XRN2	5-3’ exoribonuclease 2
YBX3	Y-box –binding protein 3
YY1	Yin Yang 1
ZFN	Zing Finger Nucleases